IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Bruce Joseph ROSER

Serial No.: 09/888,734

Filing Date: 25 June 2001

For: DRIED BLOOD FACTOR COMPOSITION COMPRISING TREHALOSE

Examiner: Francisco Chandler Prats

Group Art Unit: 1651

DECLARATION OF EDWARD G. D. TUDDENHAM

UNDER 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Edward G D Tuddenham, declare as follows:

1. I am Professor of Haemostasis at the Imperial College Faculty of Medicine, London, UK. I am familiar with the terminology used in the field of blood-derived factors, and with the nature of Factor VIII. I have been practicing in this field for thirty years. A copy of my *curriculum vitae* is attached.

2. I have reviewed the above-referenced application in its entirety. This application nowhere describes the treatment of Factor VIII with thrombin or any other protease to effect its activation. At no location is "activated Factor VIII" referred to. If such treatment had been performed, the "Factor VIII" would be referred to as "activated Factor VIII" or some additional

description would be given to indicate that the Factor VIII as it would be found in plasma, or as it would be recombinantly produced using the gene encoding that found in plasma, had been treated with a protease. I have been told that the record in this application indicates that the US Patent Office ("the Office) agrees with the foregoing, and that the Office understands the claims as presently presented are directed to methods to prepare a stable, dried composition of "native" Factor VIII, i.e., they refer to a Factor VIII other than that "activated" by thrombin treatment.

3. I have reviewed the Office action mailed 1 September 2004 and note the comments on page 6 stating that "the proteins (i.e., native Factor VIII and activated Factor VIII) possess numerous virtually identical amino acid sequences." However, substantial portions of the native form are missing from activated Factor VIII. Factor VIII is a heterodimer of a heterogeneous 90 - 210 kD heavy chain, having a 90 kD constant region and a variable region of up to 120 kD, and a light chain of 80 kD. This is the case whether the Factor VIII is in the form that circulates in the plasma, or the commercial form obtained from plasma, or the commercial form obtained by recombinant DNA techniques. However, *activated* Factor VIII is a hetero*trimer* containing only a portion of the native Factor VIII light chain (73 kD) and two fragments of the constant region of the heavy chain of 50 kD and 43 kD. Although some amino acid sequences are retained, they are rearranged and a very large portion (amounting to an average of 124 kD (out of a total heterodimeric mass of 290 kD)) is missing. Thus, almost half of the amino acid sequences of native Factor VIII are missing from activated Factor VIII.

4. Page 6 also states "at the very least, Curtis establishes generally that Factor VIII has a therapeutic utility that can be preserved upon freeze-drying in the presence of trehalose." This is not entirely accurate. Curtis concerns only preservation of activated Factor VIII, which activated Factor VIII does have therapeutic activity, while native Factor VIII does not (absent

activation by protease). The behavior of the already active molecule is not informative with respect to the behavior of the circulating heterodimeric form, which represents native Factor VIII, since that form is not active at all. Indeed it is referred to in the literature as the 'procofactor', which is analogous to the zymogen of the protease factor thrombin called prothrombin. Thus, even if Curtis showed that the activated form of Factor VIII, which itself has cofactor activity, could be stably preserved in the presence of trehalose, there is no scientific basis to extrapolate this to the native Factor VIII, which lacks such cofactor activity until proteolytically cleaved by thrombin. It simply does not follow. Great care is taken in the literature and in practice to distinguish between the inactive pro- forms of clotting factors and their activated forms as these have highly different properties. For example the activated forms are thrombogenic and have shortened half-lives *in vivo*.

5. In my opinion, the activated form of Factor VIII and the native form of Factor VIII (as a circulating heterodimer) are sufficiently different that the physical behavior of one is not predictive of the physical behavior of the other. Not only is almost half of the amino acid sequence missing in the activated form; the arrangement of the remaining peptides is different. Instead of a heterodimer with a heterogeneous heavy chain of 90-210 kD and a light chain of 80 kD, the activated form is a trimer that is not heterogeneous and comprises two segments of 50 and 43 kD, as well as a 73 kD monomer. In view of the heterogeneity of the native Factor VIII and in view of its markedly different structure from activated Factor VIII, the behavior of these materials would be expected to be very different rather than similar.

6. I have also reviewed U.S. Patent 5,364,756 to Livesey, *et al.* I have noted that claim 17, dependent on claim 1, specifies the material subjected to the process of claim 1 as Factor VIII. Claim 1 is directed to a method for preserving a suspension of biological material,

which comprises preparing a cryosolution using a suspension of biological material. With respect I submit that to chemists, pharmacologists and biologists a suspension means particulate matter held dispersed in some fluid, not a molecular substance dissolved in some aqueous solution. I am familiar with the solubility of characteristics of Factor VIII and can verify that a suspension of Factor VIII as condensed perhaps crystalline particles could reasonably be prepared only by use of extremely high concentrations that are not realistically contemplated or by denaturing the protein. The suspensions exemplified in the Livesey patent are of insoluble materials such as cells or viruses. The description of Livesey is inappropriate to Factor VIII, which is alluded to, inexplicably, in a 'shopping list' of materials that reasonably relate to Livesey's process in column 4, lines 57-64. A skilled practitioner of the art, familiar with the characteristics of Factor VIII, would understand that the inclusion of Factor VIII in such a list in this context is clearly an error or an optimistic attempt to be all encompassing and hence over inclusive.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements are made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Executed at London, United Kingdom, on 23rd November 2004.
(city) (state) (day)

E. G. D. Tuddenham
(name)

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Structure of human factor VIII

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The deduced amino acid sequence of human factor VIII, obtained from the DNA sequence, predicts a mature polypeptide of 2,332 amino acids containing a triplicated domain structure. The polypeptide has 35% sequence homology with the copper-binding plasma protein, ceruloplasmin. Determination of the thrombin cleavage sites in plasma-derived factor VIII polypeptides allows prediction of the domains involved in the associated activation and inactivation of the protein.

PREPARATIONS of factor VIII/von Willebrand factor complex[1–12] contain four closely related properties or activities: factor VIII coagulant activity, an antigen associated with the factor VIII coagulant activity, platelet adhesion promoting activity and an antigen precipitated by antisera raised against the purified complex (factor VIII-related protein). Factor VIII separated from the complex has associated trace amounts of protein, is unstable and consists of multiple polypeptide chains[13–17], hindering detailed characterization studies.

The purification of human factor VIII by affinity to a monoclonal antibody directed against the coagulant activity of factor VIII[18], has allowed characterization of the protein fragments of factor VIII or thrombin-activated factor VIII by partial amino acid sequence analysis. This sequence information has been used to isolate cDNA and genomic clones encoding human factor VIII[19,20]. The protein sequence deduced from these clones, together with an analysis of the cleavage products associated with the activation of factor VIII by thrombin, allows the assignment of thrombin cleavage sites and the identification of most of the polypeptide fragments present in highly purified factor VIII preparations. The factor VIII sequence exhibits striking homology with the plasma copper-binding protein ceruloplasmin, suggesting novel biochemical activities for factor VIII as well as a role for metal ions other than calcium in the blood coagulation cascade.

Analysis of plasma factor VIII

Preparations of human factor VIII[18] purified over 300,000-fold from plasma contained several proteins of relative molecular mass (M_r) 210,000–80,000 (Fig. 1*A*). These protein bands were not connected by disulphide links because samples analysed under non-reducing conditions gave a similar pattern (data not shown). To determine the relationship of these multiple polypeptide chains, we analysed them by tryptic peptide mapping. The preparation used in Fig. 1*B* contained a protein of M_r 240,000, producing a peptide map which did not show identity with the other proteins of the mixture (Fig. 1*B*, *a*), and has been found to be a von Willebrand factor subunit (data not shown). Peptides of M_r 90,000–210,000 all had a common tryptic map, indicating that they are derived from the same or closely related polypeptide chains (Fig. 1*B*, *b–f*). Furthermore, Western blot analysis of the factor VIII preparations demonstrated that a factor VIII-specific monoclonal antibody[18] reacted with the M_r 90,000–210,000 polypeptides (data not shown). Two very similar patterns generated by the proteins of M_r 80,000 and 70,000 had a different peptide map (Fig. 1*B*, *g*, *h*). These results demonstrate that the fragments of M_r 90,000–210,000 are structurally related and could be pooled and treated as one polypeptide chain. The protein represented by the band of M_r 80,000 (and 70,000 when present) was analysed separately.

The purified factor VIII preparations were fractionated by gel filtation on a TSK 4000SW HPLC column; analysis of the resulting fractions demonstrated the effective separation of M_r 240,000 protein, the polypeptides of M_r 90,000–210,000 and the M_r 80,000 fragment (Fig. 2*B*). We performed amino acid sequence analysis on peptides generated from the M_r 80,000 protein, the polypeptide pool of M_r 90,000–210,000 and a fragment of M_r 90,000 from limited thrombin digestion of the M_r 90,000–210,000 pool. After digestion of each sample with trypsin, the resulting peptides were separated by reverse-phase HPLC and sequenced. The peptide sequence AWAYFSDVDLEK, used to prepare synthetic DNA probes identifying factor VIII genomic DNA clones, is indicated in Fig. 2*C*.

Structure of factor VIII protein

The molecular cloning of the entire factor VIII coding region is described in an accompanying paper[19]. The 2,351-amino acid sequence for factor VIII, deduced from the nucleotide sequence of these clones, is shown in Fig. 3. The first 19 amino acids of the sequence comprise the signal sequence for factor VIII, based on peptide sequence analysis of a fragment derived from the M_r 90,000–210,000 polypeptide pool. The N-terminal sequence of this M_r 30,000 fragment, obtained as a thrombin digest product of the M_r 90,000–210,000 pool, is identical to the first 12 amino acids which follow the predicted factor VIII leader sequence (see Fig. 5). This presequence exhibits a core of 10 hydrophobic amino acids flanked by two charged residues, a structure which conforms to that observed for the leader sequences found in most secreted proteins[21]. The mature protein contains 2,332 amino acids (calculated M_r 264,763).

The availability of the complete factor VIII sequence reveals the organization and identity of the tryptic peptides obtained from the pools of separated plasma-derived factor VIII fragments. Essentially all tryptic peptide sequences determined from the M_r 90,000–210,000 protein pools are located in the amino-

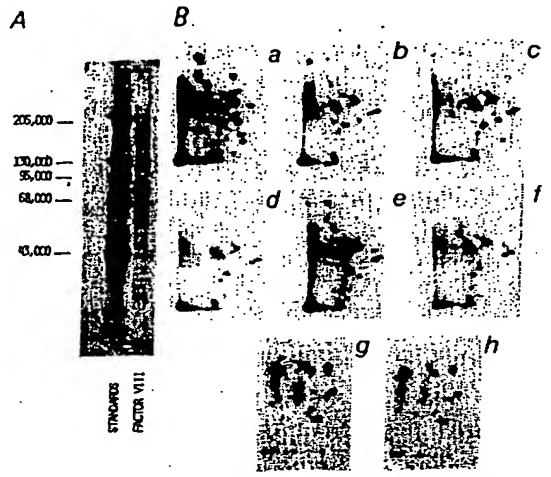

Fig. 1 *A*, Purified human factor VIII analysed by SDS-polyacrylamide gel electrophoresis. *B*, Two-dimensional tryptic mapping of factor VIII polypeptide chains. The resulting tryptic patterns of proteins of M_r: *a*, 240,000; *b*, 210,000; *c*, 170,000; *d*, 150,000; *e*, 120,000; *f*, 100,000; *g*, 80,000; and *h*, 70,000 are shown.
Methods: The purified protein[18] was reduced and analysed in a 5–10% polyacrylamide gradient gel in the presence of SDS by the procedure of Laemmli[37]. The molecular weights of the protein standards are shown (myosin, β-galactosidase, phosphorylase B, bovine serum albumin and ovalbumin). The bands were detected using the silver stain procedure of Morrissey[38]. 1 μg of a factor VIII preparation was denatured in 1% SDS and labelled with 300 μCi ^{125}I for 15 min using the iodobead procedure[38]. Labelled polypeptides were located on dried SDS-polyacrylamide gels by autoradiography and digested by incubation of gel slices with 10 μg trypsin in 0.1 M ammonium bicarbonate buffer for 6 h at 37°C. After repeated lyophilizations, peptides were dissolved in 8.8% formic acid and a portion was subjected to thin-layer electrophoresis in the same buffer (400 V for 45 min) on pre-coated TLC-cellulose plates (E. Merck, Darmstadt, FRG). For the second dimension, peptides were separated by ascending chromatography in *n*-butanol/pyridine/glacial acetic acid/water, 75:50:15:60 (v/v). The plates were then subjected to autoradiography.

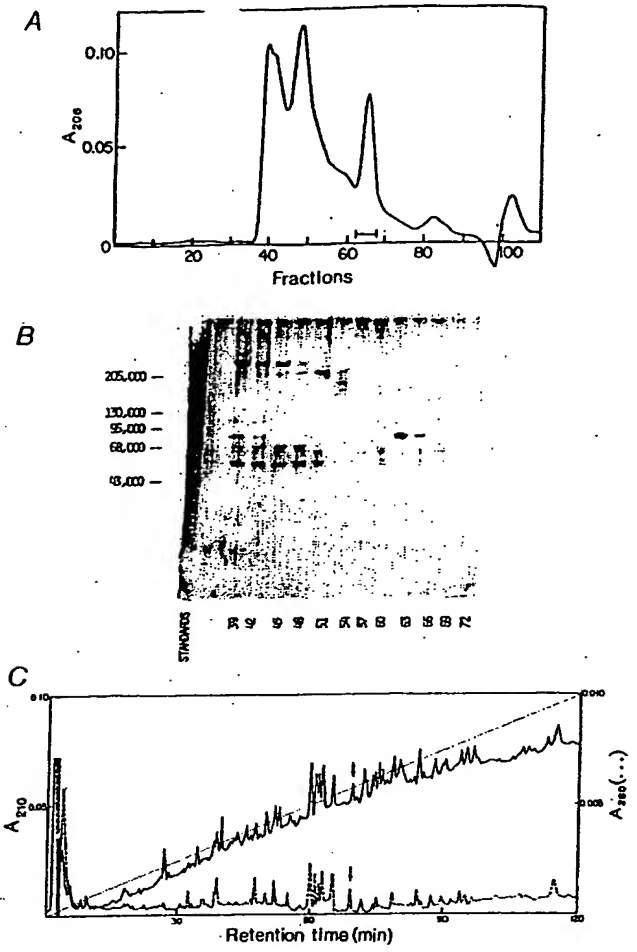

Fig. 2 Purification of factor VIII polypeptides. *A*, Fractionation of proteins by TSK 4000 HPLC. *B*, SDS-polyacrylamide gel analysis of fractions from TSK 4000 chromatography. *C*, Reverse-phase HPLC separation of tryptic peptides of M_r 80,000 protein.
Methods: *A*, Human factor VIII preparations[18] were dialysed into 1% ammonium bicarbonate containing 0.1% SDS. The samples were lyophilized and stored at −20°C until use. The samples were reconstituted in distilled water and applied to a TSK 4000 SW column (0.75×50 cm; Alltech Associates, Deerfield, Illinois) equilibrated with 0.1 M sodium phosphate buffer, *p*H 7.0, containing 0.1% SDS. Samples of ~0.15–0.25 ml were injected and the column developed isocratically at a flow rate of 0.5 ml min^{-1}. Absorbance was monitored at either 206 nm or 280 nm and fractions (0.2 ml) collected. Fractions across the profile were reduced and analysed by SDS-polyacrylamide gel electrophoresis as described in Fig. 1 legend. The protein of M_r 80,000 was pooled for tryptic digestion as shown in *A*. *C*, The TSK 4000 SW purified protein of M_r 80,000 (0.8 nM) was dialysed under a nitrogen atmosphere overnight against 0.36 M Tris-HCl, *p*H 8.6, containing 8 M urea, 3.3 mM EDTA and 10 mM dithiothreitol, DTT (final vol. 1.5 ml). The protein was alkylated by adding 15 μl of 5 M iodoacetic acid (dissolved in 1 M NaOH). The reaction was allowed to proceed for 35 min at room temperature in the dark and was quenched by adding DTT to a final concentration of 100 mM. The protein solution was dialysed against 8 M urea in 0.1 M ammonium bicarbonate for 4 h. The urea dialysis solution was changed over a period of 24 h to gradually reduce the urea concentration to a final level of 0.5 M. Trypsin was added at a weight ratio of 1:50 at 37°C for 12 h. HPLC separation of the resulting tryptic peptides was performed on a high-resolution Synchropak RP-P C-18 column (0.46×25 cm; 10 μ). The column was developed with a gradient of acetonitrile (1–70% in 200 min) in 0.1% trifluoroacetic acid. Absorbance was monitored at 210 nm and 280 nm. Each peak was collected and stored at 4°C until subjected to sequence analysis in a Beckman spinning cup sequencer with on-line phenylthiohydantoin amino acid identification[40]. The arrow identifies the peptide (AWAYFSDVDLEK) resulting in identification of a factor VIII genomic clone[19,20].

terminal half of the molecule, whereas those sequences obtained for the M_r 80,000 fragment are found at the carboxy-terminus of the factor VIII sequence (unpublished results). The most carboxy-terminal tryptic peptides identified for the M_r 90,000–210,000 pool gave the sequences GEFT and −QEE, beginning at positions 1,155 and 1,194, respectively. This shows that the M_r 210,000 fragment consists of a protein of $M_r \geq 135{,}000$ containing 14 potential asparagine-linked glycosylation sites. The location of the M_r 80,000 fragment of factor VIII is delineated by two peptide sequences which define a stretch of ~680 amino acids. The first of these was obtained from the amino-terminal sequence of the M_r 80,000 fragment beginning at position 1,649 (see Fig. 5), whereas the second corresponds to a tryptic peptide, MEVLGCEAQDL, 12 amino acids from the C-terminus predicted by the DNA sequence. Thus, there is no significant removal of C-terminal sequences from the plasma-derived molecule. The failure to recover tryptic peptide sequences from the region between position 1,200 and the M_r 80,000 fragment is probably due to the relatively low concentration of the M_r 210,000 species in the M_r 90,000–210,000 fragment pool. This position should therefore be considered the minimal C-terminal extent of the M_r 210,000 protein.

Computer-aided analysis of the factor VIII protein sequence revealed two types of internal homology: the first consists of a triplicated segment (A domain) found at positions 1–329, 380–711 and 1,649–2,019 of the mature polypeptide (Fig. 4); the second and third domains of the triplication are separated by a region of 983 amino acids (B domain) extremely rich in potential asparagine-linked glycosylation sites. In addition, an unrelated duplication of 150 amino acids is found at the C-terminus of the molecule (C domain). The A domains have ~30% amino acid homology, whereas the C domains are ~40% homologous. Most

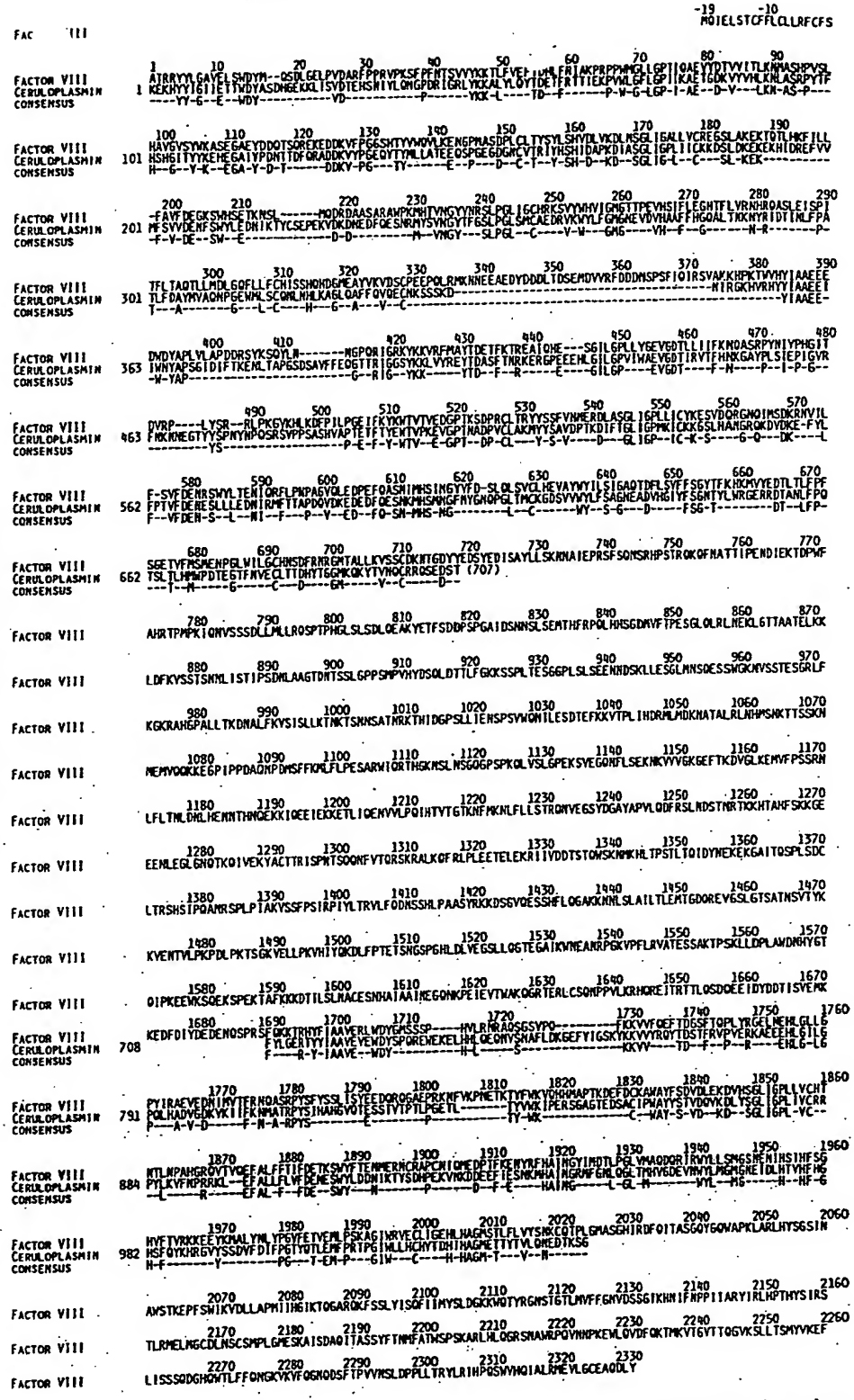

Fig. 3 Homology of human factor VIII with human ceruloplasmin. The deduced factor VIII amino acid sequence derived from cDNA and genomic clones[19,20] is compared with that of human ceruloplasmin[22]. The consensus line shows residues which are identical in the two proteins. The numbering above the sequences is that of factor VIII. The number preceding the ceruloplasmin line represents the numbering of the amino acid sequence beginning the line. The single-letter notation for amino acids is used: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

of the 23 cysteine residues of the mature polypeptide are clustered in the A and C domains and occupy similar positions Fig. 6), suggesting that the structures of both repeated domains of factor VIII reflect conserved disulphide bonding arrangements.

The A domains of the factor VIII protein show striking homology with the copper-binding plasma protein ceruloplasmin (Fig. 3). Amino acid sequence analysis of ceruloplasmin has revealed a structure consisting of three contiguous domains sharing ~30% homology[22–24]. The triplicated domains of factor VIII and ceruloplasmin exhibit a pairwise homology of 30% (Fig. 4). Although the B domain has no substantial homology with any known sequence, the C domain shares 20% amino acid homology with the discoidin lectins from *Dictyostelium*[25].

Ceruloplasmin contains six copper atoms in three distinct types of coordination; two of type 1, one of type 2 and three electron paramagnetic resonance-nondetectable type 3 copper ions[26]. The type 1 copper ions are thought to bind to the carboxy-terminal portion of the domains of ceruloplasmin (domain residues 240–350; Fig. 4) based on sequence homology with the type 1 copper-binding protein plastocyanin[27]. The four amino acid side chains proposed as the ligands for the type 1

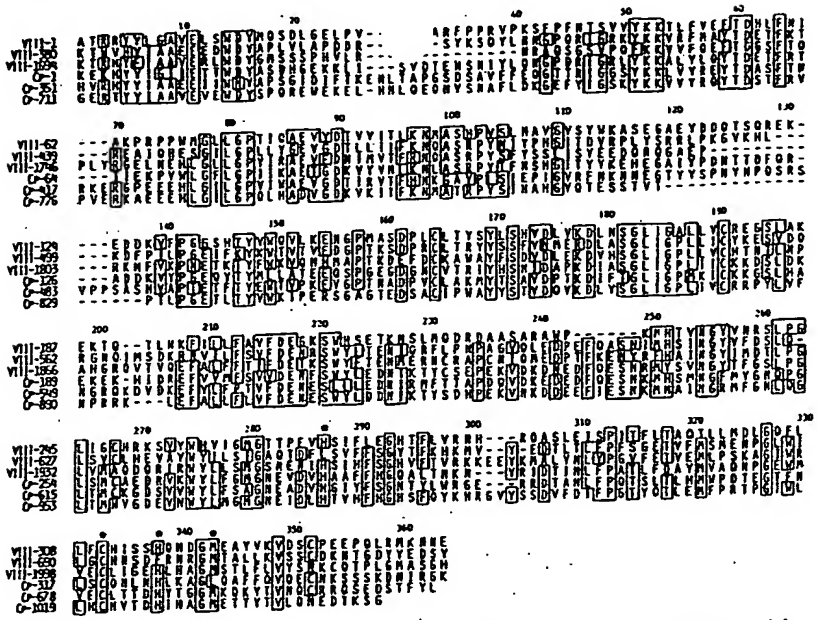

Fig. 4 Domain homology of human factor VIII and human ceruloplasmin[22]. Identical residues in four of the six domains are boxed. The protein is indicated to the left of the lines (VIII, factor VIII; Cp, ceruloplasmin) with the associated number indicating the position in the sequence of the first amino acid of the line. A domain residue numbering system is shown above the sequences. * Residues believed to be the ligands for type 1 copper (based on homology to plastocyanin[27]).

copper atoms of ceruloplasmin are found in the first and third A domains of factor VIII (histidines 287 and 338, cysteine 333 and methionine 343; see Fig. 4). The conservation of the copper-ligand residues found in ceruloplasmin strongly suggests similar metal binding characteristics for factor VIII.

Thrombin cleavage

The coagulant activity of factor VIII is increased markedly by treatment with catalytic amounts of the serine protease thrombin[28]. Thrombin activation of factor VIII is associated with a series of polypeptide cleavages[13–17]. Further incubation with thrombin leads to degradation of the protein with a concomitant loss of coagulant activity[13–17]. To understand the structural basis for these observations, the separated pools of factor VIII fragments were subjected to thrombin digestion and the resulting products characterized by SDS-polyacrylamide gel electrophoresis. Thrombin digestion of the M_r 80,000 protein resulted in a product of M_r 73,000 (Fig. 5*A*). Treatment of the polypeptide pool of M_r 90,000–210,000 led initially to the appearance of two bands of M_r 43,000 and 50,000 (Fig. 5*A*). Longer incubation with thrombin resulted in the conversion of the M_r 50,000 fragment to polypeptides of M_r 30,000 and 20,000 (data not shown). The M_r values of these thrombin digest fragments are similar to those generated by thrombin treatment of native factor VIII preparations[13,15,16]. Amino-terminal sequence analysis was performed on the separated protein chains before and after thrombin digestion. The resulting sequences are compared with the corresponding amino acid sequences deduced from the factor VIII cDNA sequence[19] in Fig. 5*B*; also shown are the potential cleavage sites found at the amino-terminus of the M_r 80,000 protein and that which separates the M_r 90,000 fragment from the carboxy-terminal portion of the M_r 210,000 protein. The sequence surrounding the latter potential cleavage site (position 740) is similar to the amino-terminal sequence of the M_r 70,000 protein (-PRSF—RH-) (Fig. 5*B*). There is no other consensus sequence that would predict the specificity of thrombin cleavage. A homologous stretch preceeding the thrombin cleavage sites for the M_r 43,000 and 73,000 proteins is observed (Fig 5*B*), but whether this homology determines thrombin specificity or simply reflects the internal duplication is uncertain. The most consistent sequence found at thrombin cleavage sites within factor VIII is an arginine residue followed by either serine or alanine. Other such sequences (-RS- or -RA-) do occur within the protein but are not cleaved, suggesting the possible involvement of secondary structure in thrombin specificity. The cleavage that frees the M_r 80,000 protein occurs at an arginine-glutamic acid sequence, probably not a thrombin-generated cleavage site. This cleavage occurs quickly and is complete within the time required for isolation of the protein. The precursor factor VIII protein therefore may be cleaved to free the M_r 80,000 polypeptide by a protease other than thrombin.

Discussion

The structure of factor VIII revealed by the amino acid sequence predicted from the cloned cDNA and the structural characterization of polypeptide fragments described here are summarized in Fig. 6. The size of the factor VIII precursor moiety is consistent with the reported isolation of single-chain M_r 330,000 protein from plasma[18] and supports the notion that the protein circulates as a high-molecular weight form that is readily cleaved in plasma and/or during isolation to a series of degradation products.

The primary structure of factor VIII exhibits three distinct types of structural domain, including a triplicated region of ~330 amino acids (A domains), a unique region of 980 amino acids (the B domain) and a carboxy-terminal duplicated region of 150 amino acids (C domains), which are arranged in the order A1–A2–B–A3–C1–C2 (Fig. 6). The A domains of factor VIII show significant homology to ceruloplasmin, consisting also of a triplicated structure of three A domains but lacking both B and C domains[22–24]. Particularly striking is the clustering of cysteine residues at similar locations within related structural domains of factor VIII (Fig. 6). The determination of disulphide pairings for ceruloplasmin[23,29] predicts two types of internal disulphide bonding arrangements for the A domains of factor VIII. The disulphide structure proposed for the C domains of factor VIII is based on the proposition that the disulphide linkgages form between the two cysteine residues found in each domain. The large B domain which separates the second and third A domains of factor VIII contains only four cysteine residues, but the presence of 19 asparagine-linked glycosylation sites suggests that this region is extensively modified by carbohydrate addition.

The activation of factor X by factor IX_a in conjunction with factor VIII is known to require calcium ions. Factor IX_a and factor X both contain γ-carboxyglutamic acid residues which are thought to be involved in calcium binding. The protein-bound calcium ions mediate the interaction of these proteins with the phospholipid surface. The homology of factor VIII with ceruloplasmin suggests the possible involvement of copper or other metal ions in the role of factor VIII in factor X activation. One possibility for the role of such metal ion involvement is suggested by the binding of lanthanide ions by γ-carboxyglutamic acid residues[30]. It is interesting to speculate that the potential copper-binding ligands of factor VIII interact with a metal ion jointly bound by the γ-carboxyglutamate

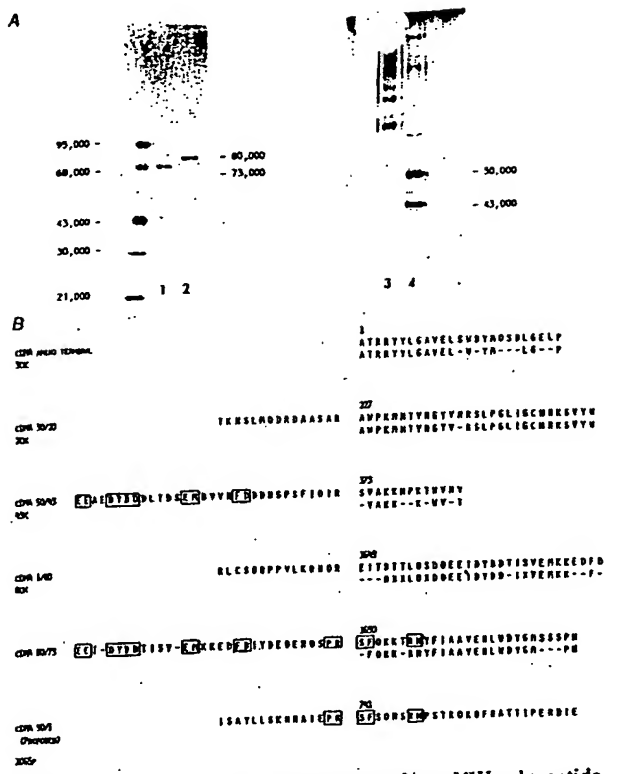

Fig. 5 *A*, Thrombin cleavage of separated factor VIII polypeptide fractions. *B*, Alignment of thrombin-generated amino termini with the deduced amino acid sequence[19].
Methods: *A*, The factor VIII polypeptides were separated as described in legend to Fig. 2*A*. SDS was removed by dialysis of the fractions against 8 M urea solutions and urea removed by dialysis against 0.01 M Tris, *p*H 8.0. Thrombin was then added at weight ratios to a maximum of 1:20. The digestions were allowed to proceed at room temperature and the extent of cleavage was monitored by SDS-gel electrophoresis. Lanes 1 and 2 are the M_r 80,000 protein with and without thrombin, respectively; lanes 3 and 4 are the M_r 90,000–210,000 protein without and with thrombin, respectively. *B*, Thrombin digestion products were separated by preparative SDS-gel electrophoresis, electroeluted by the procedure of Hunkapiller *et al.*[41] and analysed on a Beckman spinning cup sequencer[40]. The M_r 80,000 protein was obtained by TSK separation as described for Fig. 2*A*. The thrombin cleavage site is indicated by a space in the cDNA deduced sequence. The number above the subsequent amino acid corresponds to the position of that residue in the linear sequence. The amino-terminal protein sequences for the various polypeptide chains are aligned under the translated gene sequence. –, Positions where no residue could be identified; X, positions where the wrong amino acid was determined. The relative molecular masses of the proteins separated are listed (30K is the amino terminus obtained for the gel-eluted polypeptide with a M_r of 30,000); solidi indicate cleavage products (for example, 50/43 indicates the cleavage which separates the M_r 50,000 and 43,000 species). Regions which share sequence homology are boxed.

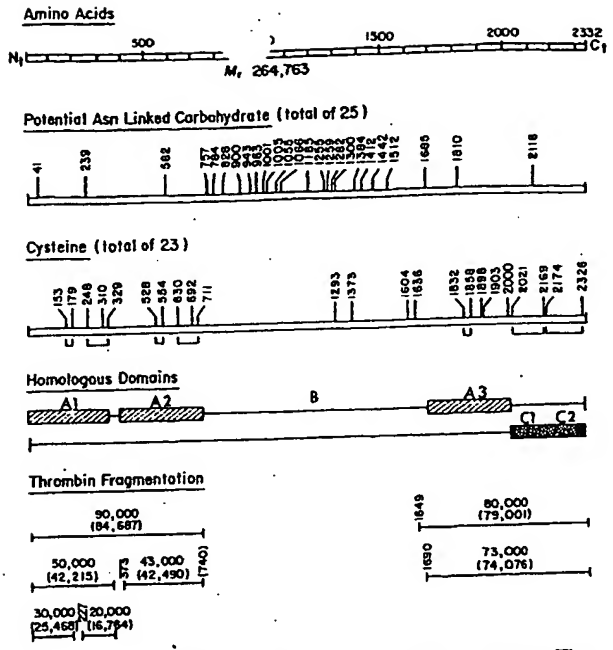

Fig. 6 Line diagram of factor VIII precursor protein. The molecular masses of the various fragments as determined by SDS-polyacrylamide gel electrophoresis are indicated (the M_r calculated from the actual amino acid composition[19] is given in parentheses). The positions of potential asparagine-linked glycosylation sites and cysteine residues are shown. The positions of the thrombin fragments and residue numbers of amino-terminal amino acids are also given. The position of cleavage of the M_r 90,000 protein has not been determined and is suggested. Boxes, the triplicated domains homologous to ceruloplasmin as well as the duplicated domain.

residues of factors IX or X. In addition to copper transport and haemostatis, several enzymatic functions have been ascribed to ceruloplasmin, including ferroxidase activity, amino oxidase activity and superoxide dismutase activity[31–33]. It will be important to determine whether any of these activities are associated with factor VIII.

The amino-terminal sequence of factor VIII thrombin fragments and the homology of factor VIII with ceruloplasmin provide insight into a functional purpose for the cleavages. Factor VIII isolated from plasma is usually degraded. In certain preparations, small amounts of a M_r 330,000 protein were observed when analysed on SDS-polyacrylamide gels run under non-reducing conditions; this protein was not observed when the factor VIII samples were reduced before electrophoresis. This reducible 330,000 may be a disulphide-linked, limited proteolytic degradation product of a single-chain M_r 330,000 factor VIII molecule first reported by Tuddenham *et al.*[18]. The portion of the protein immediately prior to the M_r 80,000 protein may thus be a disulphide linker region which holds the M_r 210,000 and 80,000 regions (or larger precursors) in the reducible 330,000 form. Exposure of plasma-derived protein preparations to thrombin results in activation of the factor VIII coagulant activity and the appearance of the band at M_r 90,000 (residues 1–740) due to the removal of the B domain or its fragments. Thrombin further cleaves both the M_r 80,000 and 90,000 proteins. The M_r 80,000 protein is cleaved after residue 1,689, releasing a M_r 4,500 peptide containing one potential asparagine glycosylation site. This peptide is highly acidic, containing 15 aspartic and glutamic acid residues and only 4 lysine or arginine residues out of a total of 41 amino acids. Thrombin cleavage of the M_r 90,000 protein to M_r 50,000 and 43,000 products occurs between the first two A domains of factor VIII after an acidic spacer region (15 aspartic and glutamic acid residues; 4 lysine/arginine; total of ~42 amino acids) having some sequence homology with the acidic peptide cleaved from the M_r 80,000 protein (Fig. 5*B*). Further cleavage of the M_r 50,000 protein (domain residue 240, Fig. 4) occurs immediately preceding the region having sequence homology with plastocyanin[27] thereby freeing this potential metal-binding domain.

Factor VIII has many properties in common with coagulant protein factor V[34–36]. These proteins function in the intrinsic coagulation cascade with an activated vitamin K-dependent coagulation protein (factors IX_a and X_a for factors VIII and V, respectively), a phospholipid surface and calcium ions. These complexes result in the specific activation of a second vitamin K-dependent coagulation protein (factor X and prothrombin for factors VIII and V, respectively). Factor VIII and factor V are both proteins of $M_r > 300{,}000$; they are cleaved by a series of thrombin-catalysed events to generate proteins of M_r 90,000 (from the amino termini) and M_r 80,000 (from the carboxy

termini) from a single-chain circul[illegible]g form. By analogy with factor V, the M_r 90,000 and 80,000 proteins would correspond to fragments D and E of factor V, respectively[34]. These two fragments of factor V can be separated from the activation peptides and isolated as a functional two-subunit protein[35,36]. Both subunits are required for factor V activity[35] and both may be required for factor VIII activity. A highly glycosylated intermediate region is cleaved from both proteins. Therefore, both factors V and VIII seem to be highly similar in structure, thrombin cleavage pattern and, presumably, function.

The studies described here provide a structural basis for defining the role of the diverse molecular forms of factor VIII in their interaction with other proteins of the coagulation cascade. The availability of complete factor VIII cDNA clones capable of programm[illegible]ecombinant factor VIII synthesis in mammalian cell cultures[19] will offer a unique opportunity to perform similar studies with the single-chain precursor molecule. The questions raised here concerning the relationship of processing events, structural domains and homology to ceruloplasmin with the biological function of factor VIII may be answered by studying structural changes introduced into the protein by modification of these cloned DNA sequences.

We acknowledge the support of Speywood Laboratories Ltd. We thank Dr John Bell, Alice Kleiss and William Henzel for laboratory assistance; Gary Hooper for his assistance in the coordination of the project between various groups and companies; and Robert A. Swanson and Dr David W. Martin Jr for their encouragement.

Received 13 August; accepted 27 September 1984.
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Molecular cloning of a cDNA encoding human antihaemophilic factor

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A complete copy of the mRNA sequences encoding human coagulation factor VIII:C has been cloned and expressed. The DNA sequence predicts a single chain precursor of 2,351 amino acids with a relative molecular mass (M_r) *267,039. The protein has an obvious domain structure, contains sequence repeats and is structurally related to factor V and ceruloplasmin.*

HAEMOPHILIA A is a bleeding disorder caused by deficiency or abnormality of a particular clotting protein, factor VIII:C[1] occurring in about 10–20 males in every 100,000. Afflicted individuals suffer episodes of uncontrolled bleeding and are treated currently with concentrates rich in factor VIII:C derived from human plasma. The available therapy, although reasonably effective, is very costly and is associated with a finite risk of infections. We report here significant progress in the use of recombinant DNA technology to provide pure human factor VIII:C as an alternative treatment for haemophiliacs.

Blood clotting begins with injury to a blood vessel. The damaged vessel wall causes adherence and accumulation of platelets activating the plasma proteins which initiate the coagulation process. Sequential activation, via specific proteolytic cleavages and conformational changes, of a series of proteins comprising the coagulation cascade eventually leads to deposition of insoluble fibrin which, together with aggregated platelets, curtails the escape of blood through the damaged vessel wall. Factor VIII:C is a large plasma glycoprotein that functions in the blood coagulation cascade as the cofactor for the factor IXa-dependent activation of factor X. It can be activated proteolytically by a variety of coagulation enzymes including thrombin[2].

In order to provide factor VIII:C for treatment of haemophiliacs we cloned a full-length cDNA. A major obstacle to the cloning effort was the large size of the protein, estimated to be at least M_r 250,000. Purification of factor VIII:C from plasma[3] is made difficult by its low abundance, its extreme sensitivity to degradation by serum proteases and its tight association with polymeric forms of the more abundant protein, von Willebrand factor. Fass *et al.*[4] have described a purification procedure for porcine factor VIII:C using monoclonal antibody

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte BRUCE JOSEPH ROSER

Appeal No. 2006-1335
Application No. 09/888,734

HEARD: August 8, 2006

Before ADAMS, MILLS and GREEN, Administrative Patent Judges.

ADAMS, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on the appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 14-16 and 20-22, which are all the claims pending in the application.

Claim 14 is illustrative of the subject matter on appeal and is reproduced below:

14. A method for preparing a stable dried composition of native Factor VIII containing a stabilizing amount of trehalose in the absence of a stabilizing amount of albumin which method comprises freeze-drying an aliquot of aqueous solution of Factor VIII containing trehalose and free of albumin.

The references relied upon by the examiner are:

Bhattacharva et al. (Bhattacharva)	5,288,853	Feb. 22, 1994
Livesey et al. (Livesey)	5,364,756	Nov. 15, 1994
Curtis et al. (Curtis)	5,824,780	Oct. 20, 1998

GROUND OF REJECTION

Claims 14-16 are rejected under 35 U.S.C. § 103(a) as being unpatentable over the combination of Curtis and Livesey.

Claims 14-16 and 20-22 are rejected under 35 U.S.C. § 103(a) as being unpatentable over the combination of Curtis, Livesey and Bhattacharva.

We reverse.

DISCUSSION

The combination of Curtis , and Livesey:

According to the examiner (Answer, page 3), Curtis describes a process of producing a stabilized preparation of activated Factor VIII[1] that is free of albumin. In this regard, the examiner finds that Curtis teaches the use of trehalose, as an alternative to albumin, to stabilize the activated Factor VIII in the preparation. Id. The examiner also finds, that Curtis teaches that the preparation of stabilized activated Factor VIII can be lyophilized for storage. Answer, bridging paragraph, pages 3-4. The examiner recognizes, however, that Curtis differs from appellant's claimed invention "by failing to describe the lyophilization of native Factor VIII." Answer, page 4.

[1] Curtis discloses the use of Factor VIII obtained from human plasma or from recombinant sources. Column 2, lines 55-63.

The examiner relies on Livesey to make up for the deficiency in Curtis. According to the examiner, Livesey "provides motivation for lyophilizing 'native' Factor VIII in trehalose without albumin by not only claiming a specific embodiment (claim 17) of lyophilizing Factor VIII[2], but also disclosing [(column 9, lines 16-24)] that trehalose, and not albumin, is one of a number of agents particularly suited to dry preservation of macromolecules such as proteins."[3] Answer, page 4.

Based on this evidence, the examiner concludes (Answer, page 5),

> the artisan of ordinary skill seeking to preserve the "native" Factor VIII encompassed by Livesey's claim 17, recognizing that Factor VIII is a protein, clearly would have looked to trehalose instead of albumin, based on Livesey's disclosure that trehalose is one of a number of agents particularly suited for protein protection in freeze-drying procedures, and albumin is not. Additional motivation for freeze-drying Factor VIII using trehalose in the absence of albumin would have been derived from the fact that the lone example of protein freeze-drying of Livesey, Example 5 at columns 23 and 24, demonstrates that the integrity of a protein containing viral vaccine is adequately protected by trehalose in buffer with no other preservative agents.

Appellant does not dispute that Curtis teaches "a method for preparing a purified and stable activated Factor VIII." Brief, page 6. Appellant argues, however, "any teaching regarding how activated Factor VIII might be stabilized

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[2] For clarity, we note that Livesey's specification and claim 17, refer to Factor VIII without reference to whether this blood factor is in its native or activated form. As we understand the examiner's argument, the examiner has interpreted this reference to Factor VIII as a generic reference to both the native and activated forms of Factor VIII.

[3] For the reasons that follow, we disagree with the examiner's interpretation of this disclosure of Livesey.

when freeze-dried is irrelevant to the behavior of native Factor VIII."[4] Id. In this regard, appellant points out that Curtis does not teach a freeze-dried preparation of native Factor VIII as required by appellant's claimed invention. Brief, page 5. With reference to the Helgerson and Tuddenham Declarations, appellant asserts that there are "considerable differences in characteristics and behavior between native and activated Factor VIII." Brief, bridging paragraph, pages 6-7. Upon consideration of the Helgerson Declaration and the Tuddenham Declaration, we find that both Declarations assert that one of ordinary skill in the art would not have a reasonable expectation of success in extrapolating the methodology applied to activated Factor VIII to the native form of Factor VIII. See e.g., Tuddenham Declaration, paragraphs 5-6; and Helgerson Declaration, paragraph 3.

In this regard, we note that Helgerson, a co-inventor on the Curtis patent, declares (paragraph 3), the work related to the Curtis patent was limited to the activated form of Factor III. According to Helgerson (id.), "[b]ecause the two protein forms are so different from one another, the attributes of, uses of, and techniques involving one may not simply extrapolated [sic] to the other." In our opinion, this is compelling insight into what a person of ordinary skill in the art would have gleaned form the disclosure of the Curtis patent.

The examiner attempts to refute the testimony set forth in the Tuddenham and Helgerson Declarations by asserting (Answer, bridging paragraph, pages 9-

[4] We recognize appellant's argument regarding the preparation of a composition that is stable without the need for refrigeration. Brief, page 6. There is, however, no specific temperature requirement in any of appellant's claims. Accordingly, we are not persuaded by this argument.

10), "[t]he fact that trehalose can be used to preserve both native and activated factor VIII demonstrates that trehalose is recognized by the art as being a cryoprotectant suitable in a number of varied applications." In this regard, we agree with Helgerson (Declaration, paragraph 3), that the examiner has overstepped the evidence on this record. While it is undisputed that Curtis discloses a method for preparing a purified and stable activated Factor VIII, as appellant points out - the evidence with regard to Livesey is not as persuasive as the examiner makes it out. According to appellant (Reply Brief, page 4), "Livesey does not teach the reader that trehalose, on its own and without albumin, can be used to stabilize Factor VIII." In this regard, we note the examiner's reliance on claim 17, and column 9, lines16-24 of Livesey to teach that the biological material comprises Factor VIII.[5] Answer, page 4. Claim 17 depends directly from claim 1 – but claim 1 does not limit the "cyroprotectant" to be used. In this regard, we direct the examiner's attention to Livesey's claim 8, which depends ultimately from claim 1 and further limits the cyroprotectant to "a vitrification solution comprising a mixture of" various chemicals. Thus, consistent with Livesey's specification, the cyroprotectants are contemplated to be used "alone or in combination with other cyroprotectants or with additional components. . . " (Livesey, column 9, lines 33-34) including albumin (Livesey, column 9, lines 5-15).

[5] Interestingly enough, the examiner does not address what is encompassed by the term "comprises" as it appears in claim 17 of Livesey, which opens the claim to the inclusion of other components including e.g., albumin. In addition, we note that the only other disclosure of Factor VIII in Livesey appears at column 4, lines 57-64, wherein Livesey discloses that "[t]he present

As we understand Livesey, the reference does not disclose or suggest that trehalose by itself can be used stabilize or preserve native Factor VIII, instead Livesey discloses (column 4, lines 5-9), "[b]y the proper selection of cryoprotective agents and the use of preselected drying parameters, almost any biological sample in suspension can be cryoprepared for a suitable desired end use." In this regard, we note that Livesey discloses (column 9, lines 5-11), "[v]arious cryoprotectants can be used in the present invention. These include . . . trehalose . . . human serum albumin and combinations thereof."

Therefore, contrary to the examiner's assertions, the evidence of record does not paint as clear a picture as the examiner would have us believe. To establish a prima facie case of obviousness, there must be both some suggestion or motivation to modify the references or combine reference teachings and a reasonable expectation of success. In re Vaeck, 947 F.2d 488, 493, 20 USPQ2d 1438, 1442 (Fed. Cir. 1991). The evidence on this record leads us to conclude that in method such as that set forth appellants' claimed invention a person of ordinary skill in the art would not have reasonably expected that trehalose, in the absence of albumin, would have stabilized native Factor VIII.

For the foregoing reasons we reverse the rejection of claims 14-16 under 35 U.S.C. § 103(a) as being unpatentable over the combination of Curtis and Livesey.

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invention can be used to preserve many different types of biological materials. It is anticipated that the method can be used to preserve materials such as . . . Factor VIII . . . ."

The combination of Curtis, Livesey and Bhattacharva:

The examiner relies on the combination of Curtis and Livesey as set forth above. According to the examiner, the combination of Curtis and Livesey does not teach the subject matter of claims 20, 21 and 22, which depend from claims 14, 15 and 16 respectively. To make up for this deficiency the examiner relies on Bhattacharva.

Appellant does not dispute the examiner's findings with regard to Bhattacharva. Instead, appellants assert (Brief, page 19), "[t]he rejection on this basis is believed in error for the same reasons as those set forth above with regard to [the combination of] Curtis and Livesey . . . ." We agree.

The examiner relies on Bhattacharva to teach "that histidine is a preferred buffer for use in Factor VIII preparations to be lyophilized." Answer, page 6. In our opinion, Bhattacharva fails to make up for the deficiency in the combination of Curtis and Livesey as discussed above. Accordingly, we reverse the rejection of claims 14-16 and 20-22 under 35 U.S.C. § 103(a) as being unpatentable over the combination of Curtis, Livesey and Bhattacharva.

SUMMARY

The rejections of record are reverse.

REVERSED

Donald E. Adams
Administrative Patent Judge

Demetra J. Mills
Administrative Patent Judge

Lora M. Green
Administrative Patent Judge

BOARD OF PATENT APPEALS AND INTERFERENCES

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1: Drugs R D. 2003;4(6):366-8. Related Articles, Links

Factor VIII--Baxter: rAHF-PFM, recombinant anti-haemophilic factor--protein-free method, recombinant factor VIII--protein-free.

[No authors listed]

Baxter Healthcare is developing a next-generation recombinant factor VIII for the treatment of haemophilia A that is produced using a totally protein-free manufacturing process. By excluding proteins or raw materials derived from human or animal sources in the final product, the risk of transmission of potentially infectious agents is removed. All of the recombinant factor VIII products currently on the market incorporate proteins or raw materials derived from either human or animal sources, as part of the nutrients required for the cells to produce the protein. Baxter has exclusive rights to Quadrant Healthcare's factor VIII stabilisation technology. Quadrant received 1 million US dollars under the agreement, and was to receive milestone payments in excess of 2 million US dollars, together with royalties on sales should a new factor VIII product using Quadrant's technology reach the market. In December 2000, Quadrant Healthcare was acquired by Elan Drug Delivery (Elan Corporation), which continued to develop the proprietary formulation and stabilisation technologies of Quadrant. In July 2003, the newly formed company Quadrant Technologies acquired Elan Drug Delivery from Elan Corporation and renamed it Quadrant Drug Delivery. In July 2003, the US FDA approved the use of ADVATE for the prevention and control of bleeding episodes in people with haemophilia A. At the Bear Stearns 16th Annual Healthcare Conference held in September 2003, Baxter reported that ADVATE was launched in August 2003 in the US. A pivotal phase III clinical trial of this recombinant factor VIII therapy, initiated in 89 previously untreated patients with haemophilia A, has been completed at sites in the US, UK, Sweden, France, Belgium, Germany, Denmark and Italy. This trial compared the pharmacokinetics, tolerability and immunogenicity of this therapy with Baxter's currently marketed recombinant factor VIII preparation, Recombinate trade mark. The first clinical data that were presented on this agent at the XXV International Congress of the World Federation of Hemophilia meeting indicate that Baxter's next-generation recombinant

factor VIII is bioequivalant with Recombinate trade mark. The two agents should have comparable efficacy. A Marketing Authorisation Application (MAA) was submitted to the EU EMEA and regulatory filings were made in Canada in September 2002. The submission was made via the centralised procedure. Should the new-generation product reach the market, Baxter will continue to offer Recombinate trade mark as well as plasma-derived factor VIII products. Preliminary study results announced in July 2003 show that factor VIII therapy was effective in controlling bleeding episodes in patients with severe and moderately severe haemophilia A who were previously treated, or had undergone surgery.

Publication Types:

- Review

PMID: 14584967 [PubMed - indexed for MEDLINE]

1: Drugs R D. 2003;4(6):366-8. Related Articles, Links

Factor VIII--Baxter: rAHF-PFM, recombinant anti-haemophilic factor--protein-free method, recombinant factor VIII--protein-free.

[No authors listed]

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Publication Types:

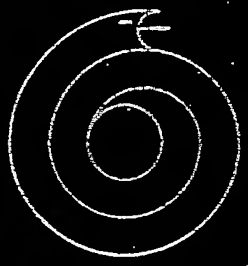
- Review

PMID: 14584967 [PubMed - indexed for MEDLINE]

VOL. 259 NOS. 1–2 18 JUNE 2003

ISSN 0378-5173
259 (1–2) 1–208 (2003)

INTERNATIONAL JOURNAL OF PHARMACEUTICS



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Review

Coagulation factor VIII: structure and stability

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Received 30 January 2003; received in revised form 20 March 2003; accepted 20 March 2003

Abstract

Factor VIII (FVIII), a coagulation factor in the blood, is one of the most complex proteins known today. To facilitate the rapid development of a more convenient and safer FVIII product and to improve the quality of life for hemophilia patients, this short article reviews the recent investigations on the structure, activity, and more importantly, stability of FVIII.

Keywords: FVIII; Structure; Stability; Formulation; Aggregation; Oxidation

1. Introduction

Factor VIII (FVIII) is an essential coagulation factor in the blood. In the complex blood clotting cascade, it serves as a co-factor for factor IXa (FIXa) in the activation of factor X (FX) to factor Xa (FXa). A deficiency or defect in FVIII is the cause of classical hemophilia (type A), a hereditary life-threatening bleeding disorder. Currently, the only therapy for this hereditary disease is life-long administration of a FVIII product. However, the limited in vivo stability of FVIII requires frequent drug administration for both preventive and therapeutic purposes and the limited in vitro stability of FVIII requires lyophilization of FVIII for long-term storage, creating inconvenience for self-administration and compromising the quality of life for hemophilia patients.

To overcome the need for frequent drug administration, FVIII gene therapy is being widely investigated for hemophilia treatment, which, if successful, may provide the ultimate therapy for hemophilia. Recent results showed that a high level of FVIII could be expressed in FVIII-deficient dogs using an adenoviral vector, but FVIII expression lasted only 5–10 days and all treated dogs developed liver toxicity, a transient drop in platelets, and anticanine FVIII antibodies (Gallo-Penn et al., 2001). While development of an effective and safe FVIII gene product is still in progress, it will likely be many years until such a therapeutic approach can be implemented in humans.

The limited in vivo/vitro stability of FVIII and slow progress in the development of a FVIII gene product is at least partly due to the relatively large size and complexity of the FVIII molecule. To facilitate the rapid development of a more convenient and safer FVIII product, this short article summarizes recent investigations on FVIII structure and stability.

2. FVIII structure and activity

In this section, we briefly discuss the complex FVIII structure and its activity, as such topics have been extensively reviewed in the past (Fay, 1993; Lollar,

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doi:10.1016/S0378-5173(03)00227-8

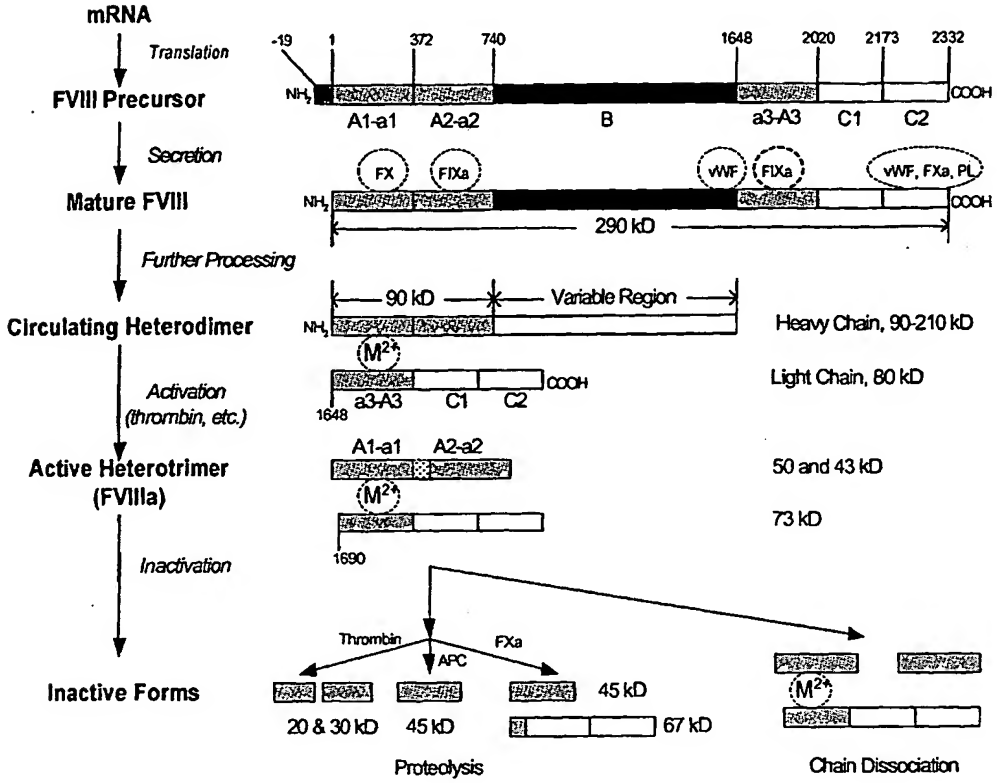

Fig. 1. FVIII structure, function, and processing. The sites of FVIII interaction with other clotting factors, vWF, phospholipids (PL), and metal ions (M^{2+}) are illustrated by dotted circles.

1995; Lenting et al., 1998). A schematic representation of FVIII structure, function and processing is shown in Fig. 1.

2.1. Primary structure

The primary structure of human FVIII was first deduced based on the DNA sequence in 1984 (Gitschier et al., 1984; Vehar et al., 1984). The X-linked FVIII gene, which has a size of 186 kb including 26 exons (Peake, 1995), encodes a polypeptide of 2351 amino acids. After processing of the signal peptide (19 amino acids), a mature FVIII molecule of 2332 amino acids is secreted with a calculated molecular weight of 264,763 Da. FVIII protein species of up to 330 kDa were observed on SDS–PAGE under non-reducing conditions, probably due to extensive glycosylation and/or disulfide crosslinking (Vehar et al., 1984). Based on homology analysis, the FVIII sequence is divided into three A, two C and a large B domain(s), abbreviated as NH_2-A1-A2-B-A3-C1-C2-COOH (Vehar et al., 1984). The three A domains are connected by three short acidic sequences: a1 (337–372); a2 (711–740); and a3 (1649–1689). Thus, a more detailed FVIII sequence is NH_2-A1-a1-A2-a2-B-a3-A3-C1-C2-COOH and the respective sequences are A1-a1: 1–372; A2-a2: 373–740; B: 741–1648; a3-A3: 1649–2020; C1: 2021-2173; and C2: 2174-2332 (Fig. 1).

Commercial FVIII concentrates showed on SDS–PAGE as mixtures of FVIII heterodimers, consisting of 90–210 kDa heavy chain (HC; A1-A2-B) and 80 kDa light chain (LC; A3-C1-C2) (Eaton et al., 1986; Bihoreau et al., 1991). Less than 1% was found to be single chain molecules on SDS–PAGE either for plasma-derived FVIII (pdFVIII) (Stoylova et al., 1999) or baby hamster kidney (BHK)-derived rFVIII (Eaton et al., 1987). The heterogeneous heavy chain

polypeptides, minimally represented by A1-A2, are derived from the 210 kDa polypeptide through proteolysis of the B-A3 junction and within the B-domain.

FVIII molecules contain many glycosylation sites and the B-domain has 19 of the 25 Asn-glycosylation sites (N-linked) (Vehar et al., 1984). Although these sites have not been completely delineated, the six Asn-glycosylation sites outside the B region are located at 41, 239, 582, 1685, 1810 and 2118 (Sandberg et al., 2001). In addition, human FVIII has multiple Ser- and Thr-glycans (O-linked) (Pittman et al., 1994; Kumar et al., 1996). Due to the complexity of extensive glycosylation and heterogeneity, the 90–210 kDa proteins of either rFVIII or pdFVIII could not be focused using several gel systems under both native and denaturing conditions, but the 80 kDa protein was focused to a pI of 6.5 and a cluster of pI's from 6.9 to 7.2 (Eaton et al., 1987).

There are six tyrosine sulfation sites in rFVIII. Three are located at 346 (HC), 1664, 1680 (LC) and the others are located at 718, 719, and 723 in the C-terminal of A2 subunit (Pittman et al., 1992; Severs et al., 1999). Although tyrosine sulfation seems to be required for normal FVIII activity, possibly by contributing to thrombin binding (Pittman et al., 1992; Pemberton et al., 1997), partial or full sulfation at Tyr718, Tyr719 and Tyr723 in B-domain-deleted two-chain FVIII did not affect the activation by thrombin (Kjalke et al., 1995). FVIII also contains a total of 23 cysteine residues and 19 of them (3 free and 8 disulfide bonded) are in the A and C domains (McMullen et al., 1995).

2.2. *Interactions between heavy and light chains*

It has long been believed that a metal ion is responsible for the association between the light and heavy chains of FVIII (Fay et al., 1986). This conclusion is based on the fact that chelation of the metal ion with EDTA leads to dissociation of the heterodimer with a concomitant loss of the procoagulant activity of a variety of FVIII forms, such as pdFVIII (Stoylova et al., 1999), rFVIII (Eaton et al., 1987) and a B-domain-deleted rFVIII (Bihoreau et al., 1991). Additional evidence for an existing metal ion in FVIII is the regeneration of 80% activity of EDTA-inactivated FVIII upon addition of excessive $MnCl_2$ (50 mM) and 30% activity for $CaCl_2$ (50 mM) (Eaton et al., 1987). A generation of 30% activity was also observed through re-association of HC and LC subunits upon addition of 25 mM Ca^{2+} (Wakabayashi et al., 2001).

Although a metal ion seems to be present in FVIII, the identity, number and role of the metal ion(s) in FVIII are still debatable. An early study with atomic absorption spectrophotometry indicated the presence of calcium in the highly-purified pdFVIII at an apparent molar ratio of 1:1 while other metals such as manganese, strontium, zinc, aluminum, magnesium, copper and iron were not present in a significant amount (<0.05 mol/subunit) (Mikaelsson et al., 1983). It was concluded that human FVIII circulates in normal plasma as a calcium-linked protein complex. In 1994, the metal was identified to be copper at a molar ratio of 1:1 in both pdFVIII heterodimers (ranging from 90/80 to 210/80 kDa dimers) and recombinant FVIII-ΔII, a B-domain-deleted FVIII (Bihoreau et al., 1994). The copper ion remained in the inactive dimer of 50/70 kDa until being released upon dissociation of this dimer, suggesting its essential role in the association of FVIII heavy and light chains. The presence of copper(I) in FVIII at a similar binding ratio was later confirmed by electron paramagnetic resonance spectroscopy (EPR) and supported by site-directed mutation studies (Tagliavacca et al., 1997). This study suggested the location of Cu(I) within the A1-domain and also demonstrated that Cu(I), not Cu(II), was able to reconstitute FVIII activity from dissociated FVIII chains. Therefore, it is possible that both copper and calcium ions are involved in HC/LC association.

In addition to metal-induced association, hydrophobic interactions between the heavy and light chains also play a significant, if not dominant, role. Two hydrophobic sites were identified by (1,1′)-bi-(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) binding in the heavy and light chains and the higher affinity site on each isolated subunit contributes to the divalent metal ion-dependent, intersubunit interaction (Sudhakar and Fay, 1996). A more recent study showed that the high-affinity hydrophobic association of the heavy and light chains (K_d = 53.8 nM) was enhanced by approximately 100-fold in the presence of 0.5 μM Cu^{2+} (Wakabayashi et al., 2001). Since Ca^{2+} at 25 mM did not affect the hydrophobic association but increased the specific activity (~60% of native FVIII activity) in this study, it appears that Ca^{2+} promotes the formation of the active conformation of

FVIII while Cu^{2+} primarily enhances the intersubunit affinity. The affinity enhancement of a metal ion may explain why low ionic strength promotes association of isolated LC and HC subunits (Donath et al., 1995).

2.3. Secondary and tertiary structure

FVIII contains mainly β-sheet structure (Sudhakar and Fay, 1998a,b; Grillo et al., 2001). A small amount of α-helices are located in the A domains (Stoilova-McPhie et al., 2002). A detailed analysis shows that B-domain-deleted rFVIII SQ contains 41% β-sheet, 14% α-helix, 26% random structure, and 19% turn by circular dichroism (CD) while the respective percentages of B-domain-deleted pdFVIII are 43, 13, 27, 18% (Fatouros and Sjostrom, 2000). In comparison, the light chain of human FVIII had 36% β-sheet, 22% α-helix, and 42% unordered structure by CD (Bihoreau et al., 1992). Activation of FVIII to FVIIIa increases significantly its β-sheet content by CD (Curtis et al., 1994).

Due to the complexity and heterogeneity of the purified FVIII, a complete high-resolution three-dimensional crystal structure of FVIII has not been determined. A few studies, however, have been conducted to characterize the overall shape of the molecule, to determine the detailed structure of its individual domains (Pratt et al., 1999), and recently, to deduce the three-dimensional structure based on low-resolution electron crystallography of two-dimensional crystals of B-domain-deleted FVIII bound to phospholipids (Stoilova-McPhie et al., 2002).

Based on the structure of nitrite reductase and ceruloplasmin, the three A domains in human FVIIIa have been modeled and the proposed structure contains 6 β-barrels (D1 through D6, two per domain) of 987 amino acids in total, roughly in a sphere size of 16 nm in diameter (Pan et al., 1995). The size is very close to what was observed for FVIII heterodimers under an electron microscope, a globular structure (contributed from A and C domains) of ~14 nm in diameter with a two-stranded tail of different lengths (contributed from B-domain) (Fowler et al., 1990), but a smaller surface coverage of 8 nm × 5 nm was observed by electron crystallography for one pdFVIII molecule when crystallized on a phospholipid membrane (Stoylova et al., 1999). The proposed model successfully explains the location of six disulfide bonds, the copper-binding site(s), the activated protein C (APC) cleavage site, and an inhibitor epitope. However, the copper-binding sites are debatable based on another modeling study (Pemberton et al., 1997).

2.4. FVIII activity

FVIII itself has minimal or no detectable procoagulant activity before proteolytic activation by thrombin or FXa (Lollar et al., 1993; Donath et al., 1995). The rate and extent of FVIII activation depend on reaction conditions, such as the relative concentrations of FVIII and thrombin or FXa. Maximal thrombin-induced activation was observed at 0.5–1 min (Sandberg et al., 2001), 2 min (Fay et al., 1986), and 10 min (Wood et al., 1984) with a corresponding potentiation of FVIII activity of approximately 20-, 13–15-, and 40-fold, respectively. As high as 40-fold (Fay et al., 1991a,b,c) or even 80-fold (Eaton et al., 1987) increase in FVIII activity was observed. Yet, the role of Ca^{2+} in the activation of FVIII by thrombin has not been clearly established (Eaton et al., 1986; Wakabayashi et al., 2001). The activated FVIII (FVIIIa) binds to FIXa at a 1:1 molar ratio (Duffy et al., 1992; Lenting et al., 1994) through multiple interactive sites in the A2 and A3 domains (Lollar et al., 1993; Fay et al., 1994; O'Brien et al., 1995; Pemberton et al., 1997; Bajaj et al., 2001). FVIIIa, FIXa (a serine protease), Ca^{2+}, and phospholipid constitute the FXase complex. FVIIIa increases the k_{cat} of FIXa-dependent conversion of FX by several orders of magnitude (Fay and Koshibu, 1998).

FVIIIa is a heterotrimer consisting of 73, 50, and 43 kDa fragments on SDS–PAGE (Vehar et al., 1984; Eaton et al., 1986, 1987). The association among the three FVIIIa subunits has not been clearly defined. Data suggest that a metal ion links the A1 and A3-C1-C2 subunits (Bihoreau et al., 1994) with a significant contribution from hydrophobic interaction (Sudhakar and Fay, 1996), while A2 is likely to interact primarily with A1 subunit ionically (Fay et al., 1991a,b,c, 1999). Because of the weak interaction between A1 and A2, the associated heterotrimer readily dissociates at physiologic pH (Fay and Koshibu, 1998). The dissociated individual subunits have less than 1% of the FVIIIa activity (Fay and Koshibu, 1998).

The large B-domain in FVIII (741–1648) does not contribute to the activity of FVIII and has no

known functions (reviewed by Fay, 1993). Several B-domain-deleted or partially deleted FVIII forms have been engineered, including $FVIII_{des\text{-}797\text{–}1652}$ (Eaton et al., 1986), $FVIII_{des\text{-}771\text{–}1666}$ (FVIII-ΔII, 165 kDa) (Bihoreau et al., 1991), and $FVIII_{des\text{-}760\text{–}1639}$ (LA-VIII) (Pittman et al., 1993). FVIII SQ, another B-domain-deleted FVIII variant in which Ser743 is linked directly to Gln1638, is now an approved commercial product (Sandberg et al., 1991, 2001). These variants do have similar clotting activity as the intact FVIII, such as $FVIII_{des\text{-}760\text{–}1639}$ (Pittman et al., 1993), and FVIII SQ (Sandberg et al., 2001). Due to their smaller molecular masses, their specific activities are generally higher than that of the full-length molecule (Eaton et al., 1987; Bihoreau et al., 1991; Sandberg et al., 2001).

3. In vivo FVIII stability

FVIII has limited in vivo stability, which is influenced by many factors, including binding to plasma proteins and antibodies, proteolytic inactivation, and non-proteolytic degradation. The half-life of high-purity FVIII in man was determined between 15 and 19 h (Ludlam et al., 1995). A single-phase in vivo decay was observed after infusion of FVIII concentrate with added calcium chloride into a hemophiliac (Foster et al., 1988).

3.1. Stabilization by von Willebrand Factor (vWF)

FVIII interacts non-covalently with vWF in plasma via at least two binding sites. A high-affinity vWF-binding site ($K_d = 2.1 \times 10^{-10}$ M) is located in A3-domain (Lys1673-Arg1689), interacting with only 1–2% of the vWF subunits (Leyte et al., 1989). The acidic region (residues 1649–1689) maintains the optimal conformation (for maximal binding) of the high-affinity vWF-binding site (Saenko and Scandella, 1997). Another vWF-binding site is located in residues 2173–2332 of the C2-domain, which also binds to phospholipids (Saenko et al., 1994; Saenko and Scandella, 1997; Jacquemin and Saint-Remy, 1998). The stoichiometry between FVIII and vWF varies depending on the study conditions (Vlot et al., 1995).

FVIII interaction with vWF results in significant stabilization and survival of FVIII in the circulation (see review by Sadler, 1998). Possible stabilization mechanisms include (1) protection of the proteolytic sites, such as Arg1689; (2) promotion of association of the heavy and light chains (Wise et al., 1991); and (3) inhibition of FVIII neutralization by FVIII antibodies (Jacquemin and Saint-Remy, 1998). These stabilization mechanisms may explain why the stability of FVIII SQ in homogenates of human subcutaneous tissue was greater in the presence of vWF (Fatouros et al., 2000) and the activity of rFVIII in immuno-depleted plasma was higher after addition of vWF (Barrowcliffe, 1994).

3.2. Effect of thrombin

Thrombin not only activates but also inactivates FVIII. Thrombin activation of either pdFVIII or rFVIII generates three major polypeptides of 73, 50, and 43 kDa on SDS–PAGE (Vehar et al., 1984; Eaton et al., 1986, 1987). Typical thrombin cleavage sites in FVIII are at Arg followed by either Ser or Ala (Vehar et al., 1984). The 73 kDa fragment comes from cleavage at Arg1689 (light chain) while the other two come from cleavages of Arg372 (between A1 and A2), and Arg740 (between A2- and B-domain) (Vehar et al., 1984; Eaton et al., 1987; Donath et al., 1995; Lenting et al., 1998). Therefore, FVIIIa is a heterotrimer of A1-a1/A2-a2/A3-C1-C2. It was noted, however, that maximum activity was actually observed some time after generation of these fragments (Eaton et al., 1986).

Thrombin-induced inactivation of FVIIIa depends on the actual study conditions, relative amount of thrombin, and the length of thrombin treatment, but not on the size of the heavy chain (Fay et al., 1986). It was demonstrated that thrombin-activated rFVIII could be stable for at least 1 h at 37 °C in one study (Eaton et al., 1987), but in a different study, treatment of human FVIII with an adequate amount of thrombin inactivated FVIII in 30 s in a buffer at pH 7.4 (Lollar et al., 1992). Even at −80 °C, loss of activity of thrombin-activated FVIII could be observed with a $t_{1/2}$ of approximately 1 week (Curtis et al., 1994).

The exact mechanism of thrombin inactivation is still not clear. At least one inactivation pathway is the conversion of 50 kDa polypeptide in FVIIIa to 20 and 30 kDa fragments but the inactivation does not corre-

late well with the formation of these two fragments by SDS–PAGE (Vehar et al., 1984). In fact, FVIIIa inactivation by thrombin was not always associated with additional cleavage (Fay et al., 1986). For example, the activity of both activated wild-type FVIII and $FVIII_{des\text{-}760\text{–}1639}$ dropped significantly 1 min after thrombin treatment but the intensity of the three major bands did not seem to change significantly in 20 min (Pittman et al., 1993). Similarly, no apparent cleavage was observed by SDS–PAGE between 5 and 22 min after thrombin treatment, but FVIIIa activity dropped in this period (Bihoreau et al., 1991). It was suggested that the non-proteolytic dissociation of the A2 subunit may be responsible for the apparent inactivation (Lollar et al., 1992).

3.3. Effect of protein C and FXa

As a feedback control for clotting, APC inactivates both FVIII and FVIIIa, while FXa both activates and inactivates FVIII like thrombin (Vehar et al., 1984; Eaton et al., 1986, 1987). APC only converts the heavy chain (90–210 kDa proteins) to a 45 kDa fragment while FXa converts the active 73 and 50 kDa fragments to 67 and 45 kDa fragments (Eaton et al., 1987). The APC-induced inactivation disrupts the interaction between A1 and A2/A3-C1-C2 subunits (Persson et al., 1995) and correlates well with the generation of the 45 kDa fragment (Eaton et al., 1986). APC-induced cleavages occur both at Arg336-Met337 (A1) and Arg562-Gly563 (A2) but it is the preferable cleavage at Arg562 that closely correlates with the loss of co-factor activity (Fay et al., 1991a,b,c; Regan et al., 1994, 1996). The binding site for APC has been localized to a region on the A3-domain of the light chain (Walker et al., 1990).

3.4. Chain dissociation—the non-proteolytic degradation

FVIII also degrades in vivo by chain (A2 subunit) dissociation—a non-proteolytic degradation mechanism, which is due to the reversible and weak interaction between A2 and the metal ion-linked A1/A3-C1-C2 dimer (Lollar et al., 1992). The chain dissociation is independent of FVIII light chain cleavage (Donath et al., 1995), but dependent on salt concentration (Fay et al., 2001) and inhibited by the presence of FIXa and phospholipid (Curtis et al., 1994). Indeed, at low salt concentrations, the FVIIIa-enhanced k_{cat} ($5.5\,min^{-1}$) for conversion of FX to FXa is approximately eight-fold greater than at a near physiological ionic strength ($0.7\,min^{-1}$) because salt interferes with the association of the A2 and A1 subunits (Fay et al., 2001). Although the relative contribution of chain dissociation to the overall inactivation of FVIII has not been determined, the higher affinity of the porcine A2 subunit for human or porcine A1/A3-C1-C2 heterodimer partly explains the higher activity of porcine FVIII relative to the human counterpart (Lollar et al., 1992; Curtis et al., 1994). Increasing the resistance to chain dissociation through mutagenesis can make FVIII more stable (Pipe and Kaufman, 1997).

4. In vitro FVIII stability

All current FVIII products are lyophilized because of its limited in vitro stability in the liquid state. This section will discuss (1) stability of FVIII in lyophilized and liquid states, during infusion, and in isolated plasma; (2) factors affecting FVIII stability; and (3) mechanisms of FVIII instability.

4.1. Stability of FVIII in lyophilized state

The stability of lyophilized FVIII products depends largely on the presence of protein stabilizers. Kogenate® and Recombinate are two representative full-length rFVIII products, that contain human serum albumin (HSA). Because of the excellent stabilizing effect of HSA, these lyophilized products are fairly stable. As much as 95% of FVIII activity can be recovered in Recombinate after storage at 30 °C for 36 months (Parti et al., 2000). To prevent potential HSA-associated pathogen exposure, the latest FVIII products are devoid of this excipient, including Kogenate® FS and ReFacto. Although a 2-year storage stability at 5–8 °C has been achieved, the overall stability of these HSA-free products is significantly compromised (Osterberg et al., 1997, 2001). An optimal formulation for lyophilized FVIII still needs to be explored with the goal of achieving a comparable stability that is afforded by HSA.

4.2. Stability of FVIII in liquid state

In contrast to lyophilized formulation, the stability of FVIII in solution is very limited. It was demonstrated by the one-stage clotting assay that as much as 10% clotting activity of the full-length rFVIII was lost in 3 days at 37 °C (Grillo et al., 2001). If rFVIII inactivation follows the Arrhenius relationship and we assume a maximum rate reduction of four times per 10 °C drop in temperature, the estimated shelf life of rFVIII at 5–8 °C is approximately 6 months, insufficient to make a commercial product. In a recent study, rFVIII SQ was shown to be stable (no loss of activity) for a year at 5 °C in a solution under nitrogen containing sucrose at 300 mg/ml and Tween 80 at 2000 ppm (Fatouros and Sjostrom, 2000). Such stability demonstrated for rFVIII SQ may result from the effects of the excipients and/or the deletion of the B-domain. The potential effect of excipients was illustrated in a study where loss of 20% FVIII activity took from less than 5 days to over 28 days at room temperature for 15 reconstituted commercial FVIII products (Martinowitz, 1994; Schulman et al., 1994). The stability difference among different FVIII products suggests strong excipient effects and that improvement of FVIII stability is feasible at least for certain products. Nonetheless, because of the limited stability of FVIII in solution, a liquid FVIII product has not been commercialized. Such a future product would greatly facilitate drug administration for patients.

4.3. Stability of FVIII during infusion

The attempt to administer FVIII by continuous infusion prompted investigations on the stability of FVIII during a simulated process. All FVIII products after reconstitution should be used within the manufacturer's recommended time period (mostly 4 h or less) for both sterility and stability reasons. However, many reconstituted products can be infused beyond this period as the stability of reconstituted FVIII is usually significantly higher than what is recommended (Martinowitz, 1994; Schulman et al., 1994; Belgaumi et al., 1999). As discussed before, FVIII stability after reconstitution varies significantly among different FVIII products. Even for the same product, the FVIII stability during infusion can be strongly affected by two additional factors—the type of delivery device and the final FVIII concentration for infusion (Martinowitz, 1994; DiMichele et al., 1996). For example, while the stability of a reconstituted FVIII concentrate (Monoclate-P®) showed no detectable degradation at room temperature in 15 days with the WalkMed 350 and CADD 1 minipump, only 70% activity was left in the Medex 2001 (Martinowitz, 1994). In another study, it was demonstrated that storing 1.4 ml of reconstituted rFVIII (Kogenate®) at 146 IU/ml in 100 ml polyvinylchloride (PVC) mini-bags for 48 h at room temperature resulted in a 42% recovery of FVIII activity but only 1.8% of activity remained when 20 ml of saline-diluted rFVIII at 2 IU/ml was stored under the same conditions (McLeod et al., 2000). The loss of FVIII activity is likely due to surface adsorption and/or surface-induced denaturation (also see Section 4.4). Therefore, inclusion of a surfactant in the reconstitution medium may be necessary to prevent the loss of protein during infusion.

4.4. Stability of FVIII in isolated plasma

Currently, there are still a significant number of pdFVIII products on the market. Therefore, proper preservation of FVIII activity in plasma is crucial for a high yield. Many stability studies have been conducted in the past but the reported stability of FVIII in plasma varied significantly. For example, approximately 54% of FVIII activity was lost in citrated plasma in 24 h at 4 °C in one study (Pepper et al., 1978), but it took the same time period at 21 °C to lose the same amount of FVIII activity in another (Cumming et al., 1987). The apparent difference in FVIII stability in plasma probably results from different concentrations of citrate used during collection, presumably due to citrate chelation of calcium ions (Krachmalnicoff and Thomas, 1983; Mikaelsson et al., 1983; Morgenthaler et al., 1985; Cumming et al., 1987; Woodhams et al., 2001). The citrate-induced detrimental effect can be reversed in a short period of time after blood collection (e.g. 4–12 h) upon recalcification (Krachmalnicoff and Thomas, 1983; Morgenthaler et al., 1985). Indeed, $CaCl_2$ stabilizes FVIII in plasma and at least 1 mM (physiological level) of $CaCl_2$ is needed to achieve a significant effect (Mikaelsson et al., 1983). Similarly, chelation of calcium by addition of >2 mM EDTA in plasma can lead to a rapid loss of activity (Krachmalnicoff and

Thomas, 1983; Mikaelsson et al., 1983; Woodhams et al., 2001) and re-addition of excessive calcium (25 mM $CaCl_2$) in citrated plasma can recover the lost activity to the control level (Krachmalnicoff and Thomas, 1983). The decay of FVIII activity in citrated plasma is non-linear (Weiss, 1965; Pepper et al., 1978; Krachmalnicoff and Thomas, 1983; Mikaelsson et al., 1983), but it does not seem to involve significant participation of proteases (Mikaelsson et al., 1983). Therefore, stabilization of FVIII in plasma does not need addition of a protease inhibitor(s) but rather, careful control of the citrate and/or calcium concentration.

4.5. Factors affecting in vitro FVIII stability

Many factors have been identified affecting in vitro FVIII stability, including temperature, presence of metal ions, salts, lipids or other formulation excipients, surface adsorption, pH, shaking, light exposure, freeze-thawing/freeze-drying, and packaging conditions.

4.5.1. Temperature

Lyophilized FVIII products tolerate thermal stress reasonably well because of the presence of a stabilizing excipient(s). Due to the stabilizing effect of HSA, 99% activity of full-length rFVIII was recovered after exposure of the lyophilized product at 40 °C for 6 months and 94% at 60 °C for 2 months (Parti et al., 2000). Dry heat treatment of pdFVIII concentrates at 80 °C for 72 h did not cause significant change in FVIII structure (Gilles et al., 1997; Raut et al., 1999). In contrast, FVIII is very sensitive to temperature change in a liquid state. For example, the rate of FVIII decay at pH 9.1 in plasma increases three-fold per 10 °C increase in the temperature range between 17 and 37 °C (Weiss, 1965). Although a higher temperature generally leads to a faster degradation of proteins, the effect of temperature on the stability of FVIII in a liquid state has not been always as predicted. A few studies have demonstrated a better stability of a few reconstituted FVIII products at room temperature than under refrigerated conditions (Saxena et al., 1991; Martinowitz, 1994; Schulman et al., 1994). Although this unusual observation has not been explained, it may have to do with reduced hydrophobic interaction at a lower temperature, as hydrophobic interaction plays a critical role in the association of HC and LC subunits. Indeed, the degradation rate of rFVIII SQ at 5 °C was faster than predicted from the Arrhenius kinetics and was suggested to be due to additional low temperature-associated chain dissociation (Fatouros et al., 1997a,b).

Like other proteins, FVIII unfolds at high temperatures in solution. Differential scanning calorimetry (DSC) analysis of rFVIII showed a major transition temperature near 58 °C in addition to several small transition temperatures below that temperature with significant aggregation above 60 °C (Grillo et al., 2001). The small transitions below 58 °C could be due to partial unfolding of FVIII subunits, as the light chain alone has a T_m value of 51 °C (Sudhakar and Fay, 1998a,b). A B-domain-deleted FVIII, rFVIII SQ, exhibits a similar thermal behavior with onset of unfolding and aggregation temperatures of about 56 and 64 °C, respectively (Fatouros et al., 1997a,b). However, detailed CD analysis showed a different thermal response in the secondary structure of the two forms of FVIII. While a conformational change was observed corresponding to increased β-sheet in rFVIII at approximately 45 °C (Grillo et al., 2001), the secondary structure of rFVIII SQ was not influenced in the temperature range of 5–55 °C (Fatouros and Sjostrom, 2000). Although a storage stability for the two forms has not been compared side-by-side, rFVIII SQ seems to be more stable in a liquid state at 5 °C than what was predicted for rFVIII (see Section 4.4), suggesting a higher conformational stability as a result of the secondary structure of rFVIII SQ.

4.5.2. Effect of metal ions

The positive effect of metal ions such as calcium on the stability of FVIII was identified in plasma (Weiss, 1965) and in a liquid or lyophilized state (Foster et al., 1988). The stabilizing effect of calcium was also observed in rapid thermal stability studies on rFVIII (Grillo et al., 2001) and storage stability studies on other FVIII forms such as rFVIII SQ (Fatouros et al., 1997a,b). Similar to Ca^{2+}, Sr^{2+} was found to stabilize FVIII in plasma (Mikaelsson et al., 1983) and rFVIII SQ in solution (Fatouros et al., 1997a,b). Other metal ions, including Cu^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}, or Ni^{2+} could not protect FVIII in plasma (Mikaelsson et al., 1983) or rFVIII SQ in solution (Fatouros et al., 1997a,b). Fe^{2+} was detrimental to FVIII SQ activity.

Although calcium ion stabilizes FVIII, excessive amounts (e.g. >50 mM Ca^{2+}) may eventually lead to destabilization of FVIII in a liquid state (Fatouros et al., 1997a,b). This is because a high calcium concentration enhances the decay of FVIIIa (Fay et al., 1993), or specifically, the dissociation of A2 and A1/A3-C1-C2 subunits (Persson et al., 1995). It was found that Ca^{2+} at 10 mM was apparently most effective in the protection of FVIII SQ in a liquid state (Fatouros et al., 1997a,b). The optimum concentration of calcium for lyophilized FVIII had not been determined.

4.5.3. Effect of salts

Salt plays a critical role in controlling both FVIII stability and solubility. Reducing the salt concentration from 58 to 18 mg/ml increased the formation of rFVIII SQ aggregates in a solution during incubation at 7 °C as determined by SEC-HPLC and also reduced its thermal aggregation temperature (Fatouros et al., 1997a,b), probably because a high ionic strength stabilizes the FVIII heterodimer (Donath et al., 1995). When the salt concentration was below 5 mg/ml (0.1 M) reversible precipitation of rFVIII SQ was observed at 125 IU/ml at pH 7, and precipitation of rFVIII SQ at a higher concentration of >1500 IU/ml occurred at a salt concentration of 9 mg/ml (0.15 M) or below (Fatouros et al., 1997a,b). Apparently, the solubility of FVIII SQ requires the presence of protein concentration-dependent amounts of salt (Osterberg et al., 2001). Probably because of this effect, increasing NaCl concentration from 9 to 19 or 35 mg/ml increased the recovery of rFVIII SQ upon freeze-drying (Osterberg and Wadsten, 1999). Since salt generally reduces the glass transition temperature of a protein formulation and inhibits the formation of hydrogen bonds with a protein during lyophilization, a mechanism is required for FVIII stabilization in a lyophilized state. The effect of salt on the stability of FVIII in a lyophilized state has not been fully elucidated.

4.5.4. Interaction with lipids

FVIII binds to phospholipids rapidly and reversibly via a multistep process (Gilbert et al., 1990; Bardelle et al., 1993). Among the phospholipids, negatively-charged phosphatidylserine (PS) and phosphatidic acid (PA) are the major FVIII-binding species (Kemball-Cook and Barrowcliffe, 1992). The interaction increases FVIII activity. For example, incubation of FVIII/vWF complex with PS and phosphatidylethanolamine (1:1) preparation can induce a two- to three-fold increase in apparent FVIII activity (Broden et al., 1983). At least 30-fold increase in the catalytic conversion of FX was observed in the presence of phospholipids (Fay et al., 2001). In addition, the interaction offers FVIII in vitro stability by inhibiting the chain dissociation (Curtis et al., 1994) or specifically, the salt-sensitive dissociation of A1 and A2 subunits (Fay et al., 2001). In a recent study, PS-containing liposomes have been shown to protect rFVIII SQ from both non-proteolytic and proteolytic degradation in homogenates of human subcutaneous tissue (Fatouros et al., 2000). Therefore, although phospholipids are unlikely to be used in a traditional FVIII product simply for stability improvement due to their limited aqueous solubility, they can be considered in designing a controlled release FVIII delivery system.

4.5.5. Surface adsorption/surfactants

FVIII adsorbs to a variety of surfaces, including glass (Martinowitz, 1994) and plastics such as PVC (DiMichele et al., 1996; Hurst et al., 1998), and polyethylene (Hurst et al., 1998). Surface adsorption of FVIII is rapid and an equilibrium can be reached within hours (DiMichele et al., 1996; Hurst et al., 1998). The amount adsorbed to a surface depends on the surface area and does not seem to be concentration-dependent (Hurst et al., 1998; McLeod et al., 2000). Therefore, the percent loss of FVIII activity would be higher when storing the same volume of FVIII solution at a lower concentration. In addition, the adsorption loss of FVIII varies depending on the composition of the FVIII product and the diluent (DiMichele et al., 1996; Parti et al., 2000). Probably because of the stabilizing effect of vWF, no loss of FVIII (recovery >95%) was observed during infusion of pdFVIII/vWF concentrate (IMMUNATE) at 50 and 100 IU/ml for 48 h through infusion pumps equipped with polyethylene, polypropylene or PVC plastic components (Thomas et al., 1999). Since the remaining FVIII activity is the only indicator for surface adsorption in these studies, any loss of FVIII activity could be not only due to surface adsorption but also to surface-induced protein denaturation. An example of surface-induced FVIII denaturation

was the rapid loss of rFVIII SQ activity at 25 °C by agitation (Fatouros and Sjostrom, 2000).

Surfactants are generally effective in reducing protein surface adsorption. It was demonstrated that polysorbate 80, a non-ionic surfactant, minimized significantly adsorption-induced loss of rFVIII SQ (Osterberg and Fatouros, 1999) and surface-induced rFVIII SQ denaturation (Fatouros and Sjostrom, 2000). In this aspect, serum albumin at 1 or 2% was not as effective as polysorbate 80 in preventing surface loss of diluted rFVIII (Recombinate) in PVC bags (Parti et al., 2000) or surface-induced rFVIII SQ denaturation (Fatouros and Sjostrom, 2000). In addition to the surface effect, limited data suggest that polysorbate 80 may stabilize FVIII in solution (Osterberg and Fatouros, 1999) and during lyophilization (Osterberg et al., 2001). Nevertheless, the role of polysorbate 80 as a FVIII stabilizer during long-term storage has not been clearly established, at least in a lyophilized state. The peroxide content in polysorbate 80 needs to be carefully controlled in future stability studies as it may adversely affect protein stability (Ha et al., 2002).

4.5.6. Effect of other formulation excipients

Many formulation excipients have been examined and a few of them clearly stabilize FVIII in a liquid state, including sucrose, sorbitol, mannitol (Fatouros et al., 1997a,b), histidine (Sandberg et al., 2001), glycine (Brodniewicz-Proba and Beauregard, 1987), and some other amino acids (Margolis and Eisen, 1984). Glycine (Brodniewicz-Proba and Beauregard, 1987), sucrose and raffinose (Besman et al., 2000) also stabilize FVIII in a lyophilized state. On the other hand, trehalose, a commonly-used protein stabilizer, failed to stabilize rFVIII SQ in solution (Fatouros et al., 1997a,b). Citrate and phosphate are detrimental to FVIII, respectively, during storage at 20 °C (Foster et al., 1988) and during the freeze-thaw process (Hynes et al., 1969), presumably due to the chelation effect and buffer-induced pH shift during freeze-thaw, respectively (Anchordoquy and Carpenter, 1996). Several antioxidants have been shown to stabilize FVIII SQ in a solution, including glutathione, acetylcysteine, methionine (Osterberg and Fatouros, 1994, 1996). Glutathione also stabilizes FVIII in a lyophilized albumin-free formulation (Besman et al., 2000).

The identification of these FVIII stabilizers has undoubtedly facilitated and will continue to facilitate the design of a stable HSA-free FVIII product. The remaining tasks are to determine the optimal stabilizing concentration of individual excipients, possible existence of any synergistic effect, and the optimal combination of these excipients.

4.5.7. Effect of pH

The solution pH strongly affects the stability of FVIII in a liquid state. The pH effect depends on the composition of the FVIII solution. While the most stable pH for FVIII in plasma was found between 6.2 and 6.7 at 37 °C (Weiss, 1965), that for pdFVIII concentrate at 4 °C was between 6.2 and 7.0 (Wolf, 1959). The most stable pH range for rFVIII SQ at 7 °C was between 6.0 and 7.0 in a solution containing 58 mg/ml NaCl and shifted slightly to 6.5–7.0 at a reduced salt concentration of 5.8 mg/ml (Fatouros et al., 1997a,b). Outside this optimal pH range, rFVIII SQ not only aggregates but also forms fragments (Fatouros et al., 1997a,b). We recently found that the optimal pH range for full-length rFVIII in a solution at 40 °C was between 6.6 and 7.0 (Wang and Kelner, 2003). Since the solution pH can strongly affect the stability of a lyophilized protein (Chang et al., 1996), it would be necessary to determine whether the optimal pH range in a solution would hold true in storing lyophilized FVIII.

4.5.8. Effect of packaging conditions

Packaging conditions may affect the stability of a protein product. This is also true for FVIII (Woodhams et al., 2001). In the evaluation of the stability at room temperature of 15 reconstituted FVIII concentrates, 6 of these showed superior stability in plastic containers, whereas 3 of these showed superior stability in glass containers (Martinowitz, 1994). These results suggest several possibilities—(1) variation in surface composition and/or properties even for the same type of containers; (2) variation in the amount and composition of container leachables; and (3) variation in the degree of FVIII/surface interaction due to different product excipients. The effect of container on FVIII stability was shown to be temperature-dependent. At 4–8 °C, 2 of 15 reconstituted FVIII concentrates showed better stability in plastic containers and 2 concentrates showed better stability in glass containers;

at 20–23 °C, most concentrates showed better stability in plastic containers; and at 37 °C, all concentrates showed equal or better stability in plastic containers (Schulman et al., 1994). Therefore, stability studies comparing different types of containers should be conducted at the product storage temperature. However, due to the limited diffusion of molecules in a solid state, containers would not be expected to play a critical role in storing lyophilized FVIII products, unless the air permeation through the container and/or container stoppers is significantly different. This is because air has been shown to accelerate the inactivation of rFVIII SQ not only in a solution (Osterberg and Fatouros, 1996; Fatouros et al., 1997a,b) but also in a lyophilized state (Osterberg et al., 2001).

4.5.9. Freeze-thawing/drying

Freeze-thaw does not seem to cause significant inactivation of FVIII in a non-phosphate buffer. It was demonstrated that freeze-thawing of rFVIII three times does not cause aggregation or loss of activity (Grillo et al., 2001). FVIII SQ was stable after ten freeze-thaw cycles in a formulation containing 65 mM His, 300 mM NaCl, 4 mM $CaCl_2$ and 250 ppm Tween 80 (Osterberg and Fatouros, 1998). However, freeze-drying caused a 10% loss of rFVIII SQ (ReFacto) activity, regardless of the formulation pH (6.0–8.0) (Osterberg et al., 2001). The results suggest that inactivation of rFVIII SQ occurred in the drying step. One possible solution is to add specific monosaccharides and/or disaccharides, which can adequately form hydrogen bonds with FVIII for possible protection against dehydration-induced protein conformation change and/or denaturation.

4.5.10. Effect of light exposure

A recent study demonstrated that lyophilized FVIII (Recombinate) both at 28 IU/ml and 103 U/ml was stable upon exposure to accelerated simulated natural light for 10 h, but the activity of reconstituted FVIII was reduced by 31 and 29% at 26 and 106 IU/ml, respectively, under the same lighting conditions (Parti et al., 2000). Light promotes formation of free radicals, which can initiate oxidative damage of proteins (Hovorka and Schoneich, 2001). Since radical-induced reaction is diffusion-controlled (Maillard et al., 1983), the rate of such a reaction in solution would be expected to be much faster than in a solid state.

4.6. Mechanisms of in vitro instability

Limited stability studies so far suggest that multiple mechanisms may be involved in the inactivation of FVIII in vitro. One of them is aggregation. It was demonstrated that incubation of rFVIII at 0.5 mg/ml in a solution at 37 °C led to formation of soluble aggregates by both SEC-HPLC (26% aggregates at day 7 with 15% loss in activity) and dynamic light scattering (size increase) (Grillo et al., 2001). The aggregation process is conformation-induced and has a 6-h lag time (nucleation process). This aggregation behavior was also demonstrated for rFVIII SQ, which underwent an initial chain dissociation (nucleation process), followed by aggregation of the dissociated heavy chain (Fatouros et al., 1997a,b). In addition, the dissociation of the LC and HC subunits of rFVIII SQ by SDS–PAGE correlated well with the loss of its activity. A FVIII mutein, $FVIII_{des\text{-}794\text{–}1689}$, devoid of one thrombin cleavage site, has enhanced in vitro stability due to its resistance to subunit dissociation (Pipe and Kaufman, 1997). Recently, we found that rFVIII aggregation was apparently the major mechanism of inactivation in solution at 40 °C and both non-covalent and covalent (disulfide-bonded) pathways of FVIII aggregation were involved (Wang and Kelner, 2003).

Kinetically, the loss of rFVIII activity was demonstrated to be first or pseudo-first order (Manning et al., 1995; Fatouros et al., 1997a,b; Wang and Kelner, 2003). Since protein aggregation arising from physical protein–protein interactions generally exhibits an apparent reaction order of ≥ 2 (Fink, 1998; De Bernardez Clark and Schwarz, 1999), the major mechanism of rFVIII aggregation is probably not a simple physical protein–protein interaction process; rather it is probably initiated and controlled by a protein conformational change (Grillo et al., 2001).

Oxidation is clearly involved in FVIII inactivation. First of all, treatment of FVIII with hydrogen peroxide inactivates FVIII rapidly in several studies (Austen, 1970; Manning et al., 1995; Stief et al., 2000). Other oxidizing and inactivating agents include iodine (Austen, 1970), sodium hypochlorite and chloramines (Stief et al., 2000). Even air could significantly accelerate the inactivation of rFVIII SQ both in a solution (Osterberg and Fatouros, 1996; Fatouros et al., 1997a,b) and in a lyophilized state (Osterberg et al., 2001). Although the oxidation sites have not

been identified, cysteine and methionine residues are likely to be involved, as air-induced oxidation can be inhibited either by methionine, glutathione, or acetylcysteine (Osterberg and Fatouros, 1996) and chloroamine T-induced oxidation of FVIII can be inhibited significantly also by methionine or cysteine in 10-fold molar excess (Stief et al., 2000). In fact, significant amount of FVIII aggregates were found to be reducible, suggesting possible oxidation of free cysteines (or disulfide exchange) (Wang and Kelner, 2003). Another line of supporting evidence for the possible involvement of free cysteines in FVIII inactivation is the rapid loss of FVIII activity upon modification of the free cysteine groups (Austen, 1970). However, not all the free cysteines are required for full FVIII activity (Manning et al., 1995).

The FVIII molecule is large and complex. Limited data suggest that residues 721–729 (Kjalke et al., 1995), and some amino and lysine groups (Manning et al., 1995) are essential for FVIII activity (Manning et al., 1995). The hemophilia database (http://europium.csc.mrc.ac.uk) reveals that mutation of FVIII can occur on a single amino acid at more than 200 sites, and the mutated FVIII is partially or completely non-functioning. Therefore, loss of FVIII activity in vitro could result from a modification of a critical sequence or even a single amino acid. Therefore, complete delineation of FVIII inactivation mechanism is a daunting task.

5. Conclusions

Although significant progress has been made in the past several decades in understanding FVIII structure and stability, our knowledge can be enhanced by further investigations. The major areas of future research include: (1) evaluation of the high-resolution three-dimensional structure of the protein and the ternary pre-coagulant complex; (2) stabilization mechanism of FVIII in both the liquid and solid states, and (3) the role of the multiple mechanisms that appear to be involved in degradation of the co-factor both in vivo and in vitro. Through further efforts, we may achieve the goal of developing a more stable, convenient, economical, and safe FVIII product to further improve the quality of life for hemophilia patients.

Acknowledgements

We are very grateful to Dr. Sheryl Martin-Moe for her critical review of the manuscript and her support of the FVIII project.

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blood

JOURNAL OF THE AMERICAN SOCIETY OF HEMATOLOGY

VOLUME 95
NUMBER 4
FEBRUARY 15, 2000

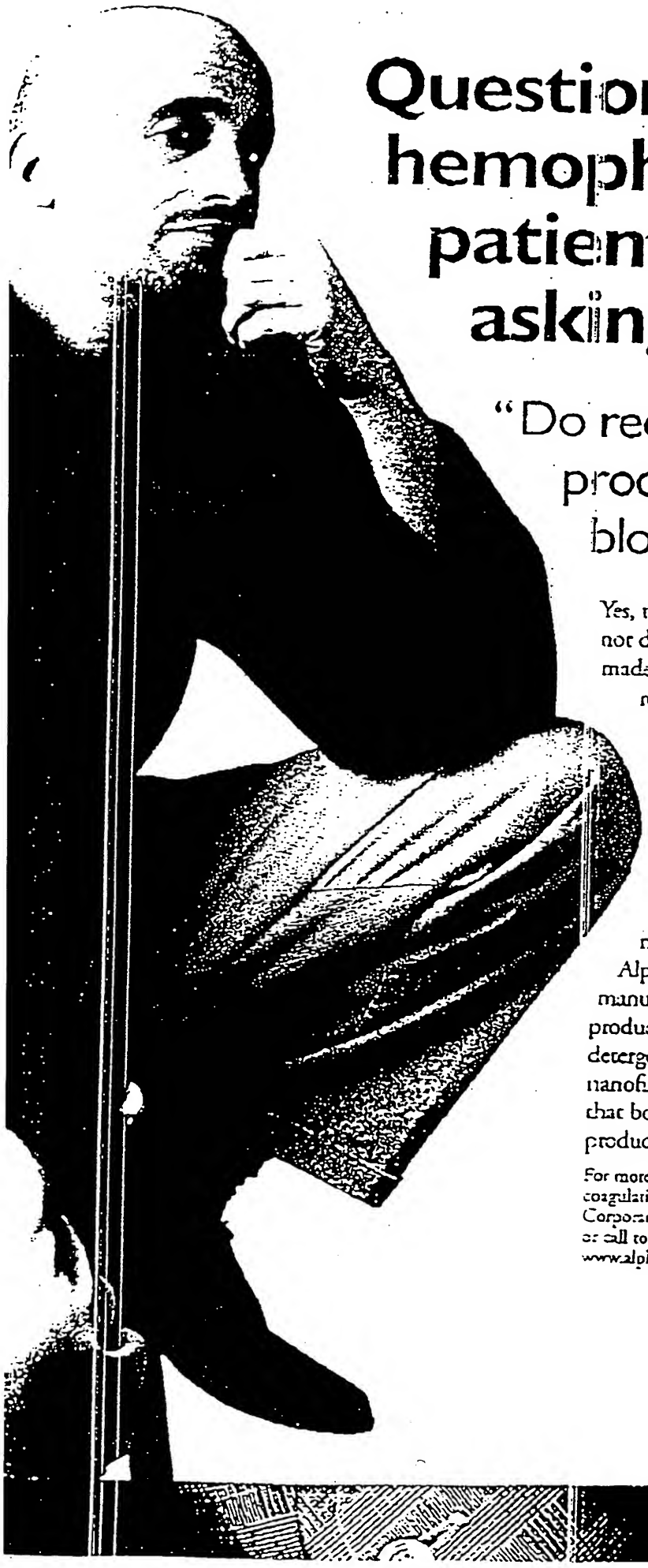
Natural anticoagulants and sepsis

Sickle cell day hospitals

PCR detection of haptoglobin anaphylaxis

P53 and CML blast crisis

Blood 5/2/00 issue



Questions hemophilia patients are asking:

"Do recombinant Factor VIII products contain human blood elements?"

Yes, they do. The factor proteins themselves are not derived from human blood. They are made from animal cells. However, all licensed recombinant Factor VIII products contain albumin, which is necessary for preserving the factor proteins in recombinant factor products. Albumin is derived from pooled human plasma, much the same way as plasma-derived factor products.

Today there are many effective safeguards against viral contamination of products made from human plasma. Safeguards Alpha Therapeutic Corporation® uses in manufacturing its plasma-derived factor products include affinity chromatography, solvent detergent treatment, heat treatment and nanofiltration. These kinds of safeguards mean that both plasma-derived and recombinant factor products are very safe and effective.

For more information about Alpha Therapeutic Corporation® coagulation factor products, write to: Alpha Therapeutic Corporation, 5555 Valley Boulevard, Los Angeles, CA 90032 or call toll free 1 (800) 292-6118 or visit our web site at www.alphather.com.

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AlphaNine®SD/Alphanate®/Profilnine®SD

ANTIHEMOPHILIC FACTOR (HUMAN)
ALPHANATE®
Solvent Detergent/Heat Treated

DESCRIPTION

Antihemophilic Factor (Human), Alphanate®, Solvent Detergent/Heat Treated, is a single dose, sterile, lyophilized concentrate of Factor VIII (AHF) intended for intravenous administration in the treatment of hemophilia A, or acquired Factor VIII deficiency.

Alphanate® is prepared from pooled human plasma by cryoprecipitation of the Factor VIII, fractional solubilization, and further purification employing heparin-coupled, cross-linked agarose which has an affinity to the heparin binding domain of vWf/FVIII:C complex.[1] The product is treated with a mixture of tri(n-butyl) phosphate (TNBP) and polysorbate 80 to reduce the risks of transmission of viral infection. In order to provide an additional safeguard against potential non-lipid enveloped viral contaminants, the product is also subjected to a 60°C heat treatment step for 72 hours. However, no procedure has been shown to be totally effective in removing viral infectivity from coagulation factor products.

Alphanate® is labeled with the antihemophilic factor potency (Factor VIII:C activity) expressed in International Units (IU) per vial, which is referenced to the WHO International Standard.

Alphanate® contains Albumin (Human) as a stabilizer, resulting in a final container concentrate with a specific activity of at least 5 IU FVIII:C/mg total protein. Prior to the addition of the Albumin (Human) stabilizer, the specific activity is significantly higher.

When reconstituted with the appropriate volume of Sterile Water for Injection, USP, Alphanate® contains 0.3 - 0.9 g Albumin (Human)/100 mL; NMT 5 mmol calcium/L; NMT 750 µg glycine/IU FVIII:C; NMT 1.0 U heparin/mL; 10 - 40 mmol histidine/L; NMT 0.1 mg imidazole/mL; 50 - 200 mmol arginine/L; NMT 1.0 µg polyethylene glycol and polysorbate 80/IU FVIII:C; NMT 10 mEq sodium/vial; and NMT 0.1 µg TNBP/IU FVIII:C.

CLINICAL PHARMACOLOGY

Antihemophilic Factor (Human) is a constituent of normal plasma and is required for clotting. The administration of Alphanate® temporarily increases the plasma level of this clotting factor, thus minimizing the hazard of hemorrhage.[2,3] Following the administration of Alphanate® during clinical trials, the mean *in vivo* half-life of Factor VIII observed in 12 adult subjects with severe hemophilia A was 17.9 ± 3.6 hours. In this same study, the *in vivo* recovery was 96.7 ± 14.5% at 10 minutes postinfusion.[4] Recovery at 10 minutes postinfusion was also determined as 2.4 ± 0.4 IU FVIII rise/dL plasma per IU FVIII infused/kg body weight.[4]

The solvent detergent treatment process has been shown by Horowitz, et al., to provide a high level of virus kill without compromising protein structure and function.[5] The susceptibility of human pathogenic viruses such as the human immunodeficiency viruses, hepatitis viruses, as well as marker viruses such as sindbis virus and vesicular stomatitis virus (VSV), to inactivation by organic solvent detergent treatment has been discussed in the literature.[6]

Gammar-P I.V.—Cont.

10) Allow the product vial to remain undisturbed for 5 minutes after diluent addition. Do not touch or mix during this time.

11) After 5 minutes, mix the product vial by gently swirling the vial without creating excessive foam. Never shake the product vial.

Note: A syrup-like layer may remain on the bottom of the vial following reconstitution. Swirl gently to disperse this layer until a homogenous solution is obtained.

12) Examine solution. All unreconstituted product should dissolve with gentle swirling and the solution should be clear and ready to administer in 20 minutes or less.

13) Product contains no preservative. Infusion must be initiated within 3 hours of reconstitution. If not used within this time frame, it should be properly disposed of and not administered.

14) Reconstituted product does not need to be filtered. If a filter is used, it should be a 15 micron filter or larger.

15) If several doses of Immune Globulin Intravenous (Human), Gammar®-P I.V., are to be pooled aseptically for administration, avoid excessive formation of foam in the pooling container and gently swirl the pooling container to mix. **DO NOT SHAKE THE POOLING CONTAINER.**

Administration

CAUTION: When entering the product stopper with an IV set spike for administration, care should be taken to follow the path made by the transfer spike (see Reconstitution).

Immune Globulin Intravenous (Human), Gammar®-P I.V., is to be administered by intravenous infusion. The infusion should begin at a rate of 0.01 mL/Kg/minute, increasing to 0.02 mL/Kg/minute after 15 to 30 minutes. Most patients tolerate a gradual increase to 0.03 – 0.06 mL/Kg/minute. For the average 70 kg person this is equivalent to 2 to 4 mL/minute. If adverse reactions develop, slowing the infusion rate will usually eliminate the reaction. Discard any unused solution.

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration whenever solution and container permit.

HOW SUPPLIED

Individual Vial Packages

Immune Globulin Intravenous (Human), Gammar®-P I.V., is supplied in single dose vials, with diluent and sterile, vented transfer spike for reconstitution. The 10 g dosage form package also contains an administration set. The following dosage forms are available:

(See table at top of previous page)

Bulk Package

Immune Globulin Intravenous (Human), Gammar®-P I.V., 5 g immune globulin/vial is supplied in a bulk pack (NDC 0053-7486-06) of six (6) single dose vials. Each single dose vial should be reconstituted with 100 mL Sterile Water for Injection, U.S.P. (not supplied).

STORAGE

When stored at temperatures not exceeding 25°C (77°F), Immune Globulin Intravenous (Human), Gammar®-P I.V., is stable for the period indicated by the expiration date on its label. Avoid freezing which may damage container for the diluent.

CAUTION: FEDERAL LAW PROHIBITS DISPENSING WITHOUT PRESCRIPTION.

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Revised: June, 1997 IBM 12173

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Antihemophilic Factor (Recombinant)
HELIXATE® R

DESCRIPTION

Antihemophilic Factor (Recombinant), HELIXATE® is a sterile, stable, purified, non-pyrogenic, dried concentrate which has been manufactured by recombinant DNA technology. HELIXATE is intended for use in therapy of classical hemophilia (hemophilia A). HELIXATE is produced by Baby Hamster Kidney (BHK) cells into which the human factor VIII (FVIII) gene has been introduced.[1] HELIXATE is a highly purified glycoprotein consisting of multiple peptides including an 80 kD and various extensions of the 90 kD subunit. It has the same biological activity as FVIII derived from human plasma. In addition to the use of the classical purification methods of ion exchange chromatography and size exclusion chromatography, monoclonal antibody immunoaffinity chromatography is utilized along with other steps designed to purify recombinant factor VIII (rAHF) and remove contaminating substances. The final preparation is stabilized with Albumin (Human) and lyophilized. The concentration of HELIXATE is approximately 100 IU/mL. The product contains no preservatives.

Each vial of HELIXATE contains the labeled amount of rAHF in international units (IU). One IU, as defined by the World Health Organization standard for blood coagulation factor VIII, human, is approximately equal to the level of factor VIII activity found in 1.0 mL of fresh pooled human plasma. The final product when reconstituted as directed contains the following excipients: 10–30 mg glycine/mL, not more than (NMT) 500 ug imidazole/1000 IU, NMT 600 ug polysorbate 80/1000 IU, 2–5 mM calcium chloride, 100–130 mEq/L sodium, 100–130 mEq/L chloride, and 4–10 mg Albumin (Human)/mL. HELIXATE must be administered by the intravenous route.

CLINICAL PHARMACOLOGY

The clinical trial of HELIXATE has included 168 patients, enrolled over a 55-month period. A total of 16,186 infusions have been utilized in this trial. The study was conducted in several stages.

Initial pharmacokinetic studies were conducted in 17 asymptomatic hemophilic patients, comparing pharmacokinetics of plasma-derived Antihemophilic Factor (Human) (pdAHF) and HELIXATE.[2] The mean biologic half-life of rAHF was 15.8 hours. The mean biologic half-life of pdAHF in the same individuals was 13.9 hours. A similar degree of shortening of the activated partial thromboplastin time was seen with both rAHF and pdAHF. The mean in vivo recovery of rAHF was similar to pdAHF, with a linear dose-response relationship. The recovery and half-life of rAHF was consistent with initial results following 13 weeks of exclusive treatment with HELIXATE. Subsequently, 826 recovery studies were conducted in 58 hemophilic patients participating in later clinical studies. Mean recovery from this group was 2.48% per IU/kg infused.

Fourteen (14) subjects from initial pharmacokinetic studies commenced home treatment with rAHF. Forty-four (44) additional subjects were then enrolled who treated themselves at home exclusively with rAHF. A total of 12,730 infusions have been administered under this portion of the study, of which 1,021 were given in clinic for recovery studies, 7,339 were given for treatment of bleeds, 4,361 were given as prophylaxis, 5 for minor surgery not requiring hospitalization, and 4 for unspecified reason.

Forty-eight (48) patients have received rAHF on 63 occasions for surgical procedures or in-hospital treatment of serious hemorrhage. Eleven (11) received rAHF for the first time in this study, while 37 were already on study or study participants under an investigation of previously untreated patients. Hemostasis has been satisfactory in all cases, with no adverse reactions.

In a study of previously untreated patients, a total of 3,254 infusions have been administered to 96 patients over a 48-month enrollment period. Hemostasis was successfully achieved in all cases.

During the analytical characterization of Antihemophilic Factor (Recombinant), HELIXATE®, analyses for carbohydrate structure revealed the presence of terminal galactose α1—3 galactose residues. Since naturally occurring antibody to this structure has been reported in humans, a trial in 18 patients was performed in which the half-life and recovery of rAHF with high levels of this carbohydrate residue was compared to that with HELIXATE, which contains low levels of this structure. As in the normal population, all patients had preexisting endogenous antibody to galactose α1—3 galactose in titers ranging from 1:320 to 1:5120. No significant change in antibody level was noted during the study. While the mean recovery for HELIXATE in the study, 2.76%/IU/kg (N = 43), was significantly different from that of rAHF with high levels of residues, 2.43%/IU/kg (N = 18), p = 0.0001, the recovery for rAHF with high levels of galactose α1—3 galactose is not significantly different from the 2.48%/IU/kg recovery obtained in the larger study from the 58 patients treated with HELIXATE mentioned above. Based on these results, the galactose α1—3 galactose residue appears to have no clinical significance.

INDICATIONS AND USAGE

HELIXATE is indicated for the treatment of classical hemophilia (hemophilia A) in which there is a demonstrated deficiency of activity of the plasma clotting factor, factor VIII. HELIXATE provides a means of temporarily replacing the missing clotting factor in order to correct or prevent bleeding episodes, or in order to perform emergency and elective surgery in hemophiliacs.

HELIXATE can also be used for treatment of hemophilia A in certain patients with inhibitors to factor VIII. In clinical studies of HELIXATE, patients who developed inhibitors to study continued to manifest a clinical response when inhibitor titers were less than 10 Bethesda Units (B.U.) per mL. When an inhibitor is present, the dosage requirement of factor VIII is variable. The dosage can be determined only by clinical response, and by monitoring of circulating factor VIII levels after treatment (see DOSAGE AND ADMINISTRATION).

HELIXATE does not contain von Willebrand's factor and therefore is not indicated for the treatment of von Willebrand's disease.

CONTRAINDICATIONS

Due to the fact that Antihemophilic Factor (Recombinant) contains trace amounts of mouse protein (maximum 30 ng/IU rAHF) and hamster protein (maximum 0.04 ng/IU rAHF), HELIXATE should be administered with caution to individuals with previous hypersensitivity to pdAHF or known hypersensitivity to biologic preparations with trace amounts of murine or hamster proteins.

Assays to detect seroconversion to mouse and hamster protein were conducted on all patients on study. No patient has developed specific antibody titers against these proteins after commencing study, and no allergic reactions have been associated with rAHF infusions. Although no reactions were observed, patients should be warned of the theoretical possibility of a hypersensitivity reaction, and alerted to the early signs of such a reaction (e.g., hives, generalized urticaria, wheezing and hypotension). Patients should be advised to discontinue use of the product and contact their physician if such symptoms occur.

WARNINGS

None.

PRECAUTIONS

General

HELIXATE is intended for the treatment of bleeding disorders arising from a deficiency in factor VIII. This deficiency should be proven prior to administering HELIXATE.

The development of circulating neutralizing antibodies to factor VIII may occur during the treatment of patients with hemophilia A. In a study of previously untreated patients, inhibitor antibodies have developed in 17 of the 92 patients (18.5%) who have had at least one follow-up titer. The incidence of antibodies is 15/56 (26.7%) in patients with severe disease (<2% factor VIII), 2/18 (11%) in patients with moderate disease (2–5% factor VIII) and 0/18 in patients with mild disease (>5% factor VIII). Ten of the antibodies were high titer (>10 Bethesda Units), three were low titer, and four were low titer and transient. Studies most closely resembling the design of the study of inhibitor development with Antihemophilic Factor (Recombinant), HELIXATE®, have reported incidences of inhibitor formation ranging between 13.4 and 52% for patients treated with pdAHF.[3–9] The incidence of inhibitor formation in previously untreated patients treated with HELIXATE appears to be consistent with that reported in the literature, however the true immunogenicity of HELIXATE is not known at present. Patients treated with rAHF should be carefully monitored for the development of antibodies to rAHF by appropriate clinical observation and laboratory tests.

Product administration and handling of the infusion set and needles must be done with caution. Percutaneous puncture with a needle contaminated with blood can transmit infectious virus including HIV (AIDS) and hepatitis. Obtain immediate medical attention if injury occurs.

Place needles in sharps container after single use. Discard all equipment including any reconstituted HELIXATE product in accordance with biohazard procedures.

Carcinogenesis, Mutagenesis, Impairment of Fertility

In vitro evaluation of the mutagenic potential of HELIXATE failed to demonstrate reverse mutation or chromosomal aberrations at doses substantially greater than the maximum expected clinical dose. In vivo evaluation of rAHF using doses ranging between 10 and 40 times the expected clinical maximum also indicated that HELIXATE does not possess a mutagenic potential. Long-term investigations of carcinogenic potential in animals have not been performed.

Pediatric Use

HELIXATE has been proven to be safe and efficacious in newborns and children while under investigation as previously treated (n=21) and previously untreated patients (n=96) (see CLINICAL PHARMACOLOGY and PRECAUTIONS).

Pregnancy Category C

Animal reproduction studies have not been conducted with HELIXATE. It is also not known whether HELIXATE can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. HELIXATE should be given to a pregnant woman only if clearly needed.

ADVERSE REACTIONS

During the clinical studies conducted in previously treated patients, 47 out of 12,932 infusions (0.36%) were associated with 58 reported minor adverse reactions. Of these, 19 reactions were local to the injection site (e.g., burning, pruritus, erythema); and 39 were systemic complaints (dizziness, nausea, chest discomfort, sore throat, cold feet, unusual taste in mouth, and slight decrease in blood pressure). In the study with previously untreated patients, 3,254 infusions have been associated with 11 minor adverse reactions (0.34%): two reports of erythema at the injection site, one of facial flushing related to the infusion, one report of diarrhea, two reports of nonspecific rash, two reports of fever, and three reports of emesis. No serious reactions have been reported, and all reactions have been self-limited.

DOSAGE AND ADMINISTRATION

Each bottle of HELIXATE has the rAHF content in international units per bottle stated on the label of the bottle. The reconstituted product must be administered intravenously by either direct syringe or drip infusion. The product must be administered within 3 hours after reconstitution.

General Approach to Treatment and Assessment of Treatment Efficacy

The dosages described below are presented as general guidance. It should be emphasized that the dosage of HELIXATE required for hemostasis must be individualized according to the needs of the patient, the severity of the deficiency, the severity of the hemorrhage, the presence of inhibitors, and the factor VIII level desired. It is often critical to follow the course of therapy with factor VIII level assays. The clinical effect of HELIXATE is the most important element in evaluating the effectiveness of treatment. It may be necessary to administer more HELIXATE than would be estimated in order to attain satisfactory clinical results. If the calculated dose fails to attain the expected factor VIII levels, or if bleeding is not controlled after administration of the calculated dosage, the presence of a circulating inhibitor in the patient should be suspected. Its presence should be substantiated and the inhibitor level quantitated by appropriate laboratory tests. When an inhibitor is present, the dosage requirement for rAHF is extremely variable and the dosage can be determined only by the clinical response.

Some patients with low titer inhibitors (<10 B.U.) can be successfully treated with factor VIII without a resultant anamnestic rise in inhibitor titer.[1] Factor VIII levels and clinical response to treatment must be assessed to insure adequate response. Use of alternative treatment products, such as Factor IX Complex concentrates, Antihemophilic Factor (Porcine) or Anti-inhibitor Coagulant Complex, may be necessary for patients with anamnestic responses to factor VIII treatment and/or high titer inhibitors.

Calculation of Dosage

The in vivo percent elevation in factor VIII level can be estimated by multiplying the dose of rAHF per kilogram of body weight (IU/kg) by 2%. This method of calculation is based on clinical findings by Abildgaard et al.[3] and is illustrated in the following examples:

(See first table above)

(See second table above)

or

(See third table above)

(See fourth table above)

The dosage necessary to achieve hemostasis depends upon the type and severity of the bleeding episode, according to the following general guidelines:

Mild Hemorrhage

Mild superficial or early hemorrhages may respond to a single dose of 10 IU per kg,[4] leading to an in vivo rise of approximately 20% in the factor VIII level. Therapy need not be repeated unless there is evidence of further bleeding.

Moderate Hemorrhage

For more serious bleeding episodes (e.g., definite hemarthroses, known trauma), the factor VIII level should be raised to 30–50% by administering approximately 15–25 IU per kg. If further therapy is required, a repeat infusion can be given at 12–24 hours.[10]

Severe Hemorrhage

In patients with life-threatening bleeding or possible hemorrhage involving vital structures (e.g., central nervous system, retropharyngeal and retroperitoneal spaces, iliopsoas sheath), the factor VIII level should be raised to 80–100% of normal in order to achieve hemostasis. This may be achieved with an initial rAHF (Antihemophilic Factor (Recombinant), HELIXATE®) dose of 40–50 IU per kg and a maintenance dose of 20–25 IU per kg every 8–12 hours.[11, 12]

Surgery

For major surgical procedures, the factor VIII level should be raised to approximately 100% by giving a preoperative dose of 50 IU/kg. The factor VIII level should be checked to assure that the expected level is achieved before the patient goes to surgery. In order to maintain hemostatic levels, repeat infusions may be necessary every 6 to 12 hours initially, and for a total of 10 to 14 days until healing is complete. The intensity of factor VIII replacement therapy required depends on the type of surgery and postoperative regimen employed. For minor surgical procedures, less intensive treatment schedules may provide adequate hemostasis.[11, 12]

Prophylaxis

Factor VIII concentrates may also be administered on a regular schedule for prophylaxis of bleeding, as reported by Nilsson et al.[13]

Reconstitution

Vacuum Transfer

1. Warm the unopened diluent and the concentrate to room temperature (NMT 37°C, 99°F).
2. After removing the plastic flip-top caps (Fig. A), aseptically cleanse the rubber stoppers of both bottles.
3. Remove the protective cover from the plastic transfer needle cartridge with tamper-proof seal and penetrate the stopper of the diluent bottle (Fig. B).
4. Remove the remaining portion of the plastic cartridge, invert the diluent bottle and penetrate the rubber seal on the concentrate bottle (Fig. C) with the needle at an angle.

   Alternate method of transferring sterile water: With a sterile needle and syringe, withdraw the appropriate volume of diluent and transfer to the bottle of lyophilized concentrate.
5. The vacuum will draw the diluent into the concentrate bottle. Hold the diluent bottle at an angle to the concentrate bottle in order to direct the jet of diluent against the wall of the concentrate bottle (Fig. C). Avoid excessive foaming.
6. After removing the diluent bottle and transfer needle (Fig. D), swirl continuously until completely dissolved (Fig. E).
7. After the concentrate powder is completely dissolved, withdraw solution into the syringe through the filter needle which is supplied in the package (Fig. F). Replace the filter needle with the administration set provided and inject intravenously.
8. If the same patient is to receive more than one bottle, the contents of two bottles may be drawn into the same syringe through a separate unused filter needle before attaching the vein needle.

(See figures A-F in next column)

Rate of Administration

The rate of administration should be adapted to the response of the individual patient, but administration of the entire dose in 5 to 10 minutes or less is well tolerated. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit.

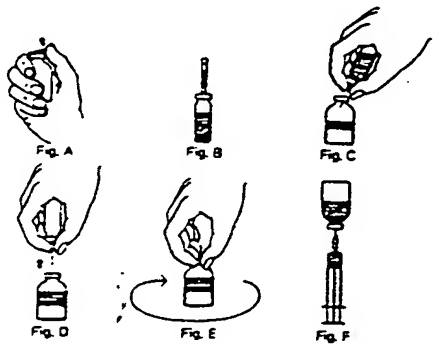
Expected % factor VIII increase = $\frac{\text{\# units administered} \times 2\%/\text{IU/kg}}{\text{body weight (kg)}}$

Example for a 70 kg adult: = $\frac{1400 \text{ IU} \times 2\%/\text{IU/kg}}{70 \text{ kg}} = 40\%$

Dosage required (IU) = $\frac{\text{body weight (kg)} \times \text{desired \% factor VIII increase}}{2\%/\text{IU/kg}}$

Example for a 15 kg child: = $\frac{15 \text{ kg} \times 100\%}{2\%/\text{IU/kg}} = 750 \text{ IU required}$

Product Code	Approximate Factor VIII Activity	Diluent
NDC 0053-8120-01	250 IU	2.5 mL
NDC 0053-8120-02	500 IU	5 mL
NDC 0053-8120-04	1000 IU	10 mL



HOW SUPPLIED

Antihemophilic Factor (Recombinant), HELIXATE® is supplied in the following single use bottles with the total units of factor VIII activity stated on the label of each bottle. A suitable volume of Sterile Water for Injection, USP, a sterile double-ended transfer needle, a sterile filter needle, and a sterile administration set are provided.

(See fifth table above)

STORAGE

HELIXATE should be stored under refrigeration (2°–8°C; 36°–46°F). Storage of lyophilized powder at room temperature (up to 25°C or 77°F) for 3 months, such as in home treatment situations, may be done without loss of factor VIII activity. Freezing should be avoided, as breakage of the diluent bottle might occur. Do not use beyond the expiration date indicated on the bottle.

CAUTION

U.S. federal law prohibits dispensing without prescription.

LIMITED WARRANTY

A number of factors beyond our control could reduce the efficacy of this product or even result in an ill effect following its use. These include improper storage and handling of the product after it leaves our hands, diagnosis, dosage, method of administration, and biological differences in individual patients. Because of these factors, it is important that this product be stored properly, and that the directions be followed carefully during use.

No warranty, express or implied, including any warranty of merchantability or fitness is made. Representatives of the Company are not authorized to vary the terms or the contents of the printed labeling, including the package insert for this product, except by printed notice from the Company's headquarters. The prescriber and use of this product must accept the terms hereof.

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